Iap2 is required for a sustained response in the *Drosophila* Imd pathway

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Abstract

Fruit flies have effective immune response against Gram-negative bacteria. Upon infection, early JNK-signaling pathway mediated response is followed by the action of the Immune deficiency (Imd) signaling cascade, a *Drosophila* equivalent of mammalian TNF-receptor pathway, leading to the release of antimicrobial peptides. Recently, Tak1-binding protein 2 (Tab2) and Inhibitor of apoptosis 2 (Iap2) were identified as components of the Imd pathway. In this study, we carried out a genome-wide kinetic analysis of the role of Tab2 and Iap2 for immune response in *Drosophila* S2 cells using oligonucleotide microarrays. *Tab2* RNAi abolished the induction of all immune response genes in S2 cells indicating its requirement for signaling both via the Imd and the JNK pathway. The role of *Iap2* was more specific. Kinetic analysis indicated that Iap2 is required to sustain antimicrobial peptide gene expression in S2 cells. Furthermore, inactivation of Iap2 by RNAi resulted in impaired microbial resistance in *Drosophila* in vivo.

Keywords: NF-κB pathway; Signal transduction; Innate immunity; *Drosophila*; Inhibitor of apoptosis2

1. Introduction

*Drosophila melanogaster* has emerged as a productive model to study conserved signaling cascades regulating immune response. There are two major pathways for microbe recognition in flies leading to production of antimicrobial peptides (AMPs): the Toll pathway which mainly responds to Gram-positive bacteria and fungi, and the Immune deficiency (Imd) pathway, which responds to Gram-negative bacteria (reviewed in [1–3]). Imd pathway is evolutionarily conserved and it corresponds closely to mammalian TNF receptor
signaling pathway. The Imd pathway signaling is mediated through PGRP-LC [4–6] to a death-domain containing protein Imd [7]. The exact signaling events occurring downstream of Imd are currently unclear, but it is known that at least the IκB kinase (IKK) complex, dFADD, Dredd and Tak1 (TGFβ-activated kinase1) are required for Relish activation. Of these, Tak1 has been implied in the sequential activation of the c-Jun N-terminal kinase (JNK) and the Imd pathways [8–11]. However, recently it was shown that although needed for AMP production, Tak1 is not required for Relish nuclear translocation in S2 cells [12] or in vivo [13], proposing a more complex regulation of Relish activity than previously suggested.

Recently, a Drosophila homologue of TAK1-binding proteins 2 and 3 (Tab2) was identified and shown to be required for AMP gene expression [12,14] and JNK phosphorylation [14]. In mammals, it has been proposed that TAB2 and TAB3 act as adaptor proteins in TRAF6, TRAF2 and TAK1 interaction [15], which leads to phosphorylation and subsequent activation of Tak1 [16]. In addition, it has been suggested that ubiquitination is needed for the activation of Tak1 and the IKK complex [17]. In mammals, TRAF proteins are known to act as K63-ubiquitin E3 ligases in NF-κB signaling, but in Drosophila Imd signaling, the E3 ligase required has not been identified. Some conflicting data have been published about the role of Trafs in Drosophila immunity [18] (reviewed in [3]). However, current reports about knockdown of all Drosophila Trafs in S2 cells [19] as well as the resistance of Traf2 mutant flies to Erwinia carotovora septic injury [20] suggest that Trafs do not participate in Imd signaling. Instead, a putative E3 ligase Iap2, a member of the Inhibitor of apoptosis protein family, was recently identified as a new component of the Imd pathway in Drosophila [12,21]. In Drosophila, there are two IAP-proteins: DIAP1 and Iap2. Both of them contain BIR (baculovirus IAP repeat) domains and a RING (really interesting new gene) domain, which is a signature for an E3 ligase. DIAP1 regulates the cell death machinery by inhibiting caspases and is hence essential for survival [22]. In mammals, there are several IAPs (reviewed in [23]), out of which the best characterized are cellular proteins cIAP1 and cIAP2 [24] and X-chromosome-linked IAP (XIAP) [25]. Although the best known function of IAPs is caspase inhibition and therefore anti-apoptotic action, they are also able to bind other molecules and change their activity by ubiquitination [23]. The exact mechanism how Iap2 and Tab2 affect the Imd pathway signaling in Drosophila is unknown. In this study, we have further characterized the role of Iap2 and Tab2 in the Imd pathway signaling. In particular, we have analyzed the kinetics of the AMP gene expression in S2 cells and in vivo, and evaluated the role of Iap2 for normal resistance in vivo.

2. Materials and methods

2.1. Cell culture

S2 cells were cultured in Schneider medium (Sigma) + 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 25°C.

2.2. Synthesis of dsRNAs

dsRNAs were produced using targeted primers (see Supplemental Table S1) and cDNA from S2 cells as a template. First the selected region of the gene was amplified using the outer primers. The obtained PCR product was used as a template for nested PCR with primers containing binding sites for T7 RNA polymerase. pMT/BiP/V5-His/GFP plasmid (Invitrogen) was used as a template for the production of the negative control GFP dsRNA. dsRNA synthesis was performed by in vitro transcription using T7 MegaScript RNA polymerase (Ambion) and the PCR products containing T7 sites as templates.

2.3. Luciferase reporter assay

Attacin A (AttA)-luciferase [26] reporter construct was kindly provided by Prof. Jean-Luc Imler and Prof. Jean-Marc Reichhart. Luciferase reporter assay for Imd pathway was performed essentially as described earlier [12,27,28]. Briefly, S2 cells were transfected with AttA-luciferase or Drs-luciferase reporter together with dsRNAs, using Fugene® transfection reagent (Roche) according to manufacturer’s instructions. Heat-killed Escherichia coli was added to the cells 48 h after transfection to induce the Imd pathway. The cells were harvested in Passive lysis buffer (Promega) at 0 (uninduced), 1, 4, 8, or 24 h after induction with heat-killed E. coli and stored at −20°C until analyzed. Luciferase values were measured using standard procedures.
2.4. Genome-wide analysis of mRNA levels using oligonucleotide microarrays

0.5 × 10⁶ S2 cells were seeded on 24-well plates and treated with 4 µg of control (GFP) or experimental dsRNAs. 48 h later heat-killed E. coli was added and cells incubated with the bacteria for 0 (uninduced), 0.5, 1, 4, 24 or 32 h. Cells (~2 × 10⁶ cells/well) were harvested and total RNA extracted using RNeasy Mini Kit (Qiagen CA, USA). Gene expression analysis was performed using the Affymetrix (Santa Clara) Drosophila Genechip Expression Analysis Technical Manual.

2.5. Semi-quantitative RT–PCR from S2 cells

5 × 10⁵ S2 cells were seeded onto 24-well plates and treated with 3 µg of experimental or control dsRNA. 48 h later, antimicrobial peptide expression was induced by heat-killed E. coli treatment. At selected time points after induction, ranging between 0 and 72 h, cells were harvested in TRIzol® Reagent (Invitrogen) and stored at –80°C until total RNA extraction according to manufacturer’s instructions. cDNA syntheses and PCR reactions for Attacin D (AttD) were carried out using SuperScript™ II One-Step RT-PCR with Platinum® Taq (Invitrogen) kit. Primers and sizes of PCR products are listed in Supplemental Table S1.

2.6. Fly stocks

Canton S and Relishnull flies were obtained from Prof. Dan Hultmark from the Umeå University. GAL4 driver C564-GAL4, UAS-RNAi transgenic fly stocks Iap2-IR and dFADD-IR as well as recently described Iap2 null mutant flies [20] were kindly provided by Prof. Bruno Lemaitre, Prof. Ryu Ueda and Dr. François Leulier. The generation of the RNAi transgenic fly stocks is described elsewhere [12,29]. The C564-GAL4 driver expresses GAL4 in adult fat body and hemocytes. C564-GAL4 flies were crossed with UAS-RNAi flies and the adult flies carrying one copy of the GAL4 driver and one copy of the UAS-RNAi construct were used in infections.

2.7. RNA extraction from flies and semi-quantitative RT-PCR

Canton S, Relishnull, and Iap2 flies were infected 0, 1, 4 or 8 h prior to RNA extraction by pricking them with a needle dipped into concentrated culture of Enterobacterium cloacae (E. cloacae). 20 flies (10 males and 10 females) were collected at each time point and snap-frozen in dry ice. The flies were homogenized in TRIzol® Reagent (Invitrogen) and the total RNAs were extracted according to manufacturer’s instructions. Semi-quantitative RT-PCR was performed using SuperScript™II One-Step RT-PCR with Platinum® Taq (Invitrogen) kit. Primers and PCR product sizes are listed in Supplemental Table S1.

2.8. Survival assay

Bacterial infections were performed by pricking adult flies with a thin tungsten needle dipped into a fresh, concentrated culture of bacteria. The survival of the flies was monitored for 72 h. The Gram-positive and Gram-negative bacteria applied were Micrococcus luteus (M. luteus) and E. cloacae, respectively.

3. Results

3.1. Iap2 activity determines the duration of AttD expression in S2 cells

Iap2 was recently identified as an essential component of the Drosophila Imd pathway [12,21]. In our earlier study, Iap2 RNAi robustly reduced the AttA-luciferase reporter activity (98±1% decrease) in response to heat-killed E. coli, whereas the effect was clearly more moderate on endogenous CecA1 gene expression as shown by semi-quantitative RT-PCR [12]. We hypothesized that this difference might be due to different AMPs studied and/or different time points used in these experiments: in the luciferase reporter assays we had used a 24 h E. coli-treatment to induce the Imd pathway, whereas a 6 h time point was used in RT-PCR reactions.

To study our hypothesis, we performed experiments where the Imd pathway was induced with heat-killed E. coli, and the expression of selected AMP genes was measured at various time points after induction, using both a luciferase reporter assay and a semi-quantitative RT-PCR assay. As shown in Fig. 1a, dsRNA-treatments targeting Rel, Tab2, Iap2, Dredd or key completely abolished the E. coli-induced AttA-luciferase activity at all time points measured. Similar results were obtained with CecA1-luciferase and Drs-luciferase reporters.
(Supplemental Fig. 1). In addition, Tab2 and Tak1 dsRNA-treatments almost entirely blocked the *E. coli*-induced endogenous *AttD* induction as measured by semi-quantitative RT-PCR (Fig. 1b). Intriguingly, endogenous *AttD* mRNA was induced comparably in *iap2* dsRNA-treated S2 cells compared to *GFP* dsRNA-treated controls at 4 h (Fig. 1b). In later time points however, *AttD* mRNA levels were markedly reduced in *iap2* dsRNA-treated cells compared to *GFP* dsRNA-treated controls (Fig. 1b). Incidentally, the expression of *AttA* in S2 cells was not sufficient to perform RT-PCR experiments. In conclusion, *Iap2* appears necessary for sustained AMP response, but it is not needed for early response in S2 cells.

**Drosophila** JNK pathway is required for a number of processes, including wound healing [30], development, apoptosis and immunity (reviewed in [31]). During immune challenge with Gram-negative bacteria, the JNK pathway is activated prior to the IKK/Relish dependent Imd pathway in S2 cells [9,10]. In S2 cells, the early response of JNK pathway signaling is terminated approximately after 4 h [10], after which the Imd pathway and consequently a different set of target genes are activated. Tak1 and Tab2 are necessary for both the Imd and the JNK pathway activity in *Drosophila* [8–10,14]. As neither Tak1 nor Tab2 are required for Relish cleavage [12,13], it is plausible that the role of these proteins in the regulation of

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**Fig. 1.** *Iap2* is required for sustained AMP gene expression in S2 cells. (a) and (c), S2 cells were transfected with *AttA-luciferase* reporter plasmid together with shown dsRNAs. Imd pathway was induced with heat-killed *E. coli*, and luciferase values measured from cells collected at given time points after induction. Luciferase values were normalized to uninduced *GFP* controls. Data is shown as mean ± SD, N = 2. (b) and (d), S2 cells were treated with indicated dsRNAs and 48 h later the Imd pathway was induced as above. Cells were collected at given time points after induction, total RNA extracted with TRIzol® and *Attacin D* and *Actin 5C* expression levels measured by RT-PCR.
the Imd pathway activity is mediated via the JNK pathway.

To test this, we investigated the role of the JNK pathway downstream components (Hemipterous (JNKK), Basket (JNK) and Kayak (homologue of human Fos; part of the API-complex)) to AMP gene expression using both AttA-luciferase reporter assay and AttD semi-quantitative RT-PCR. As shown in Fig. 1c, hep, bsk and kay RNAi moderately reduced the E. coli-induced AttA-luciferase activity compared to GFP dsRNA-treated controls in S2 cells. Similar results were obtained using semi-quantitative RT-PCR estimation of the endogenous AttD mRNA levels. These results are in line with our earlier results indicating that the JNK pathway has a role in regulating AMP expression in S2 cells [12,28]. However, Tak1 and Tab2 are clearly much more important for the AMP response than the downstream JNK pathway components. This indicates that the function of Tak1 and Tab2 is not restricted to regulation of the JNK pathway activity in S2 cells.

3.2. Iap2 is required for maintaining the expression of sustained immune response genes at genome level in S2 cells

To obtain a genome-wide view to the role of Tab2 and Iap2 in regulation of the immune response genes in S2 cells, we used oligonucleotide microarrays. S2 cells were exposed to heat-killed E. coli for 0, 0.5, 1, 4, 24, and 32 h and thereafter the mRNA levels of ~18,500 transcripts were measured using Affymetrix Drosophila Genechips. The genes whose induction with heat-killed E. coli is greater than two-fold in 4 h can be grouped into two groups based on the Relish RNAi result at the 4 h time point: Relish-dependent or sustained (n = 32), and Relish-independent or early (n = 14) response gene groups (Table 1). Others have also shown this time-dependent clustering of immune response genes [9,10]. As shown in Fig. 2a, at 4 h after E. coli induction, Relish RNAi completely eliminates the induction of sustained immune response genes. Likewise, imd, Tak1 and Tab2 RNAi block this induction, whereas Iap2 RNAi has no effect (Fig. 2a). This indicates that Imd, Tak1, Tab2 and Relish are required for induction of all the Imd pathway target genes in S2 cells, whereas Iap2 is not required at the 4 h time point. The inhibitory action of all the dsRNA treatments was analyzed and was found to be effective (Supplemental Fig. 2).

The early immune response is mediated via the JNK pathway and it does not require Relish activity (Fig. 2b) [9,10]. Relish RNAi at the 4 h time point actually increased and/or prolonged the induction of the early group genes by nearly three-fold compared to GFP dsRNA-treated samples (Fig. 2b). This demonstrates that Relish induction is required for termination of the early response as shown earlier by Park and coworkers [10]. In contrast, the induction of early response genes is almost completely blocked by Tak1 and Tab2 RNAi (Fig. 2b) indicating that Imd/Relish and Imd/JNK pathways branch at the level of Tak1 and Tab2 as shown earlier [9,10]. The effect of Iap2 RNAi to the induction of the early response group genes (Fig. 2b) was modest (29 ± 34% decrease). Therefore it appears that Iap2 has minor role in the JNK pathway signaling which is in line with recent results by Huh et al. [32].

Next, we monitored the E. coli-mediated induction of gene expression in the sustained response gene group as a function of time. The fold induction of Attacin B (AttB; the most strongly induced gene at 4h) in S2 cells treated with indicated dsRNAs is shown in Fig. 2c. In Tak1 and Tab2 dsRNA-treated cells, a marginal induction of AttB can be seen at 4 h but this induction is quickly returned to baseline levels. In contrast, in Iap2 dsRNA-treated cells, the induction at 4 h is greater than in the GFP dsRNA-treated controls, but by 32 h this induction is reduced to less than half of the controls. The same was observed when the induction levels of all the sustained response group genes (n = 32) were analyzed together (Fig. 2d). These results confirm at the genome level that Iap2 is required to maintain the expression of the sustained immune response genes in S2 cells.

Of note, the expression levels of AttA, Attacin C and Diptericin, genes whose expression has been shown to be induced in vivo in response to E. coli infection [33,34], were not markedly induced with E. coli in S2 cells. This indicates that activated Relish alone is not sufficient to induce these genes in S2 cells but another, yet unidentified factor, is required.

3.3. Impaired expression of AMPs in Iap2 knockout flies

To rule out the possibility, that in S2 cells the time-dependent effect of Iap2 RNAi on AMP expression was caused by leftover Iap2 protein, we monitored the expression levels of AttA, AttD and
CecA1 in Iap2 mutant flies that fail to express Iap2 [20]. In Fig. 3, the expression patterns of these AMPs at different time points after septic injury with E. cloacae are shown in wild-type (Canton S), Relish mutant (RelishE20) and Iap2(-/-) flies. In Iap2 null flies, the level of AttA expression is extremely
low at all time points, whereas AttD and CecA1 are clearly produced especially during the first hours after infection as measured by semi-quantitative RT-PCR (Fig. 3). This is in line with our results in S2 cells (Fig. 1b) and indicates that at least certain AMP genes can be expressed independently of Iap2 in the early phases of infection. Unexpectedly, moderate levels of AttA expression are present in Relish<sup>E20</sup> flies suggesting the existence of a Relish-independent means to induce AttA expression. Corresponding levels of AttA expression were also observed in Relish<sup>E20</sup> larvae naturally infected with Erwinia carotovora by Leulier and coworkers [20 Fig. 5C].
3.4. Iap2-IR flies are susceptible to Gram-negative bacteria

To examine the importance of Iap2 to Drosophila immunity, we tested whether Iap2 in vivo RNAi mutant (Iap2-IR) flies were susceptible to Gram-negative or Gram-positive bacteria. Canton S, RelishE20, dFADD-IR and Iap2-IR flies were infected with either M. luteus or E. cloacae (representing Gram-positive or Gram-negative bacteria, respectively). None of the tested fly strains was susceptible to M. luteus (Fig. 4a). Likewise, Canton S wild-type flies were resistant to E. cloacae. As expected, in vivo RNAi knockdown of dFADD—a known component of the Imd signaling pathway—lead to significantly reduced resistance. Iap2-IR flies behaved comparably to the dFADD-IR flies (Fig. 4b). 44 h after E. cloacae infection, only 35% of Iap2-IR flies were alive. RelishE20 flies were, as expected, very sensitive to E. cloacae. These results indicate that Iap2 is required for normal immune response in Drosophila.

4. Discussion

Both the Imd and the JNK pathways regulate the immune response against Gram-negative bacteria in Drosophila. During immune challenge, the JNK pathway is activated prior to the activation of the Imd pathway [9,10]. It has been proposed that the AP1 complex, the main target of JNK pathway activation, inhibits the activation of the Relish dependent genes [35]. This could explain the relatively slow kinetics of AMP gene expression after rapid cleavage and nuclear localization of Relish. On the other hand, Relish and IKK activities are required for terminating the expression of the JNK pathway target genes (Fig. 2b) [10]. The exact mode of regulation of these pathways is unclear. It has been shown that Tak1 and Tab2 are required for the JNK pathway activation [9,10,13,14]. The role of the JNK pathway in the expression of the AMP genes is more controversial [9–13,28,34]. It was recently reported by Delaney and coworkers [13] that the role of Tak1 in AMP release is mediated via the JNK pathway in Drosophila in vivo. In their study, Tak1 mutant immune response phenotype was rescued by over-expression of a downstream JNKK, proposing that JNK pathway activation is sufficient to induce AMP gene expression in vivo.

In our current study, we have tested the effect of RNAi knockdown of Tak1 and Tab2 and of the JNK pathway downstream components on AMP
gene expression in S2 cells. We observed a much more moderate effect with hep, bsk and kay dsRNA-treatments compared to those of Tak1 and Tab2. Therefore, the JNK pathway components appear to have a role in the AMP gene expression in S2 cells, but Tak1 and Tab2 are much more important to the AMP gene response than the downstream JNK pathway components. We conclude that the function of Tak1 and Tab2 is not restricted to JNK pathway regulation in S2 cells. We hypothesize that Relish activity is regulated both by cleavage and phosphorylation and that these two may be separate events. Tak1/Tab2 complex has no role in Relish cleavage [12,13], but it is possible that the complex functions as an IKK kinase—as the human TAK1 kinase complex [36]—regulating the IKK activity and thus phosphorylation of Relish.

The controversial reports about the involvement of the JNK pathway in AMP gene expression may be in part due to diverse times of induction used in different studies. It seems that the JNK pathway requirement for AMP gene expression is time-dependent: as shown in Fig. 1d, the expression of AMP genes appears normal within the first few hours after immune challenge in S2 cells treated with dsRNAs targeting JNK pathway (Fig. 1d). However, dsRNA treatments targeting JNK pathway components clearly decrease the AMP gene expression from 24 h onwards (Fig. 1d) [12,28].

Iap2 is required for sustained AMP gene response and therefore appears as a unique molecular switch determining the duration of the AMP gene expression in S2 cells. Correspondingly, Iap2 null mutant flies were able to express AttD and CecA1 during the first hours after infection. This indicates that at least certain AMP genes can be expressed independently of Iap2 in the early phases of infection. Curiously, we were unable to detect any AttA expression in Iap2 mutant flies. This suggests that the role of Iap2 to the induction of different AMP genes in vivo varies.

Fig. 5. Schematic representation of the JNK and Imd pathways in Drosophila immune response. (a) Early response, (b) Sustained response. Dashed line illustrates translation; asterisk (*) represents Relish putative phosphorylation. CG15678 is a potential negative regulator of the JNK and Imd pathways [28]. Question mark (?) indicates that the exact mechanism of action remains to be characterized.
How *Drosophila* Iap2 functions is yet unclear, but it can be hypothesized that the mechanism involves changing the activity of a component of the Imd pathway by ubiquitination perhaps in a similar fashion as TRAF2 and TRAF6 function in mammals. It has been suggested that an E3 ligase analogous to human TRAF2 or TRAF6 is needed in *Drosophila* to function with Bendless (Ubc13) and dUEV1a (UEV1a) in the activation of Tak1 and the IKK complex [17]. However, when we knocked down *bendless*, *dUEV1a*, *Traf1* or *Traf2* in S2 cells by RNAi, we observed no marked effect on AMP response as measured by *AttA-luciferase* reporter assay, *AttD* RT-PCR and *AttD* qRT-PCR. So in contrast to mammalian NF-κB signaling, we are unable to demonstrate any role for Ubc13, UEV1a or Traf5 in the Imd pathway signaling in S2 cells using dsRNA-treatment either alone or in combination (Supplemental Fig. 3). Others have also demonstrated that knocking down all *Drosophila* Traf5 by RNAi has no effect on Imd signaling in S2 cells [19], and that *Traf2* mutant flies are resistant to Gram-negative bacteria [20]. Therefore other yet unidentified factors are most likely involved in the Imd pathway signaling. Our current model of the Imd and JNK pathways is presented in Fig. 5.

It has been proposed that JNK pathway inactivation is caused by the proteasomal degradation of Tak1 [10], and that the E3 ligase responsible for Tak1 ubiquitination and subsequent degradation is Plenty of SH3s (POSH) [37]. As Iap2 is a putative E3 ligase, we hypothesized that it might play a role in the degradation of Tak1. However, we could not demonstrate Tak1 degradation when the Imd pathway was activated either with heat-killed *E. coli* or by Relish overexpression: Tak1-V5 signals from cells collected at the same time points with or without *E. coli* did not differ and no potential degradation products were seen. Likewise, overexpression of either wild type *Relish* or active form of *Relish (RelΔS29-S45)* did not cause decreased stability of Tak1-V5 (Supplemental Fig. 4). Furthermore, we did not see a stabilizing effect upon knockdown of *Iap2* by RNAi (data not shown).

In conclusion, the Imd signaling appears more complex than previously thought. There are many similarities to mammalian immunity pathways, but there are some important differences. For example, the roles of TRAFs, Ubc13 and UEV1a in immune challenge in *Drosophila* appear to differ from those of the mammalian counterparts. The exact molecular mechanisms of Iap2, Tak1 and Tab2 still remain elusive and are targets for further investigation.

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**Appendix A. Supplementary materials**

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dci.2007.01.004.

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