The Caspase-8 Homolog Dredd Cleaves Imd and Relish but Is Not Inhibited by p35

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Background: During the Drosophila immune response, both Imd and Relish are cleaved in a manner dependent on the caspase-8 homolog Dredd.

Results: Dredd cleaves Imd and Relish, but not the caspase inhibitor p35, without interdomain autoprocessing.

Conclusion: Imd and Relish are direct substrates for full-length Dredd.

Significance: Dredd is similar to some mammalian initiator caspases, which can function without interdomain cleavage.

In Drosophila, the Imd pathway is activated by dianopimelic acid-type peptidoglycan and triggers the humoral innate immune response, including the robust induction of antimicrobial peptide gene expression. Imd and Relish, two essential components of this pathway, are both endoproteolytically cleaved upon immune stimulation. Genetic analyses have shown that these cleavage events are dependent on the caspase-8 like Dredd, suggesting that Imd and Relish are direct substrates of Dredd. Among the seven Drosophila caspases, we find that Dredd uniquely promotes Imd and Relish processing, and purified recombinant Dredd cleaves Imd and Relish in vitro. In addition, interdomain cleavage of Dredd is not required for Imd or Relish processing and is not observed during immune stimulation. Baculovirus p35, a suicide substrate of executioner caspases, is not cleaved by purified Dredd in vitro. Consistent with this biochemistry but contrary to earlier reports, p35 does not interfere with Imd signaling in S2* cells or in vivo.

The Imd pathway is one of two NF-κB signaling pathways controlling antimicrobial peptide (AMP) induction in the Drosophila immune response. Diaminopimelic acid-type peptidoglycan (PGN), from Gram-negative and some Gram-positive bacteria, stimulates the Imd pathway through receptors PGRP-LC and PGRP-LE, and leads to the activation of Relish, a NF-κB precursor protein similar to mammalian p100 and p105. Unlike p100 or p105 processing, Relish is activated by endoproteolytic cleavage, and then translocates to the nucleus where it drives robust (100-fold or more) AMP gene expression (recently reviewed in Refs. 1 and 2).

In this pathway, the Imd protein plays a central role as receptor proximal adapter (3). Imd interacts directly with both PGRP-LE receptors (4), as well as with the Drosophila FADD homolog, which in turn recruits the caspase-8-like Dredd (5, 6). Upon PGN stimulation, Imd is endoproteolytically cleaved, at aspartate residue 30 within a caspase recognition site. Genetic studies demonstrated that this signal-induced cleavage is dependent on Fadd and Dredd. Once cleaved, Imd exposes a new N terminus that contains inhibitor of apoptosis binding motif. The E3 ubiquitin ligase Iap2 associates with cleaved Imd through the newly exposed inhibitor of apoptosis binding motif, and rapidly conjugates Imd with K63-linked polyubiquitin. K63 polyubiquitin chains are suggested to function as a scaffold to activate Tak1 and IKK kinases (Ird5 and Kenny), which are critical for Relish activation and AMP gene induction (7).

In the absence of immune stimulation, Relish is found in the cytoplasm in its full-length form, but upon immune stimulation, Relish is cleaved at aspartate 545 within a preferred caspase target site. Subsequently, the N-terminal fragment containing the Rel-homology domain translocates to the nucleus to act as a transactivator. Again, genetic data show that this cleavage requires Dredd and Fadd (8–10).

Caspases, including Dredd are cysteine proteases that play crucial roles in apoptosis and inflammation. Mammalian caspases can be sorted into two subgroups based on their functions. Apoptotic caspases integrate intrinsic and extrinsic signals and execute apoptosis, whereas inflammatory caspases cleave pro-inflammatory cytokines, processing them into their mature forms (reviewed in Refs. 11 and 12). The Drosophila genome is known to encode seven caspases. Three of them, Nedd2-like caspase (Nc)/Dronc, Dredd, and Drem/Stra1 have long prodomains typical of apical or initiator caspases. Four others (Ice, Dcp1, Damn, and Decay) have short prodomains, characteristic of executioner caspases (13). Nc, the fly homolog of mammalian caspase-9, is an initiator caspase with a single caspase-recruitment domain that is responsible for most cell death along with the executioner caspases, Ice
and Dcp1 (reviewed in Ref. 14). Dream/Strica has a novel serine/theronine-rich prodomain, and plays redundant roles with Nc in certain apoptotic contexts (15, 16). Dredd includes a prodomain with two Death effector domains and is similar to mammalian caspase-8 (17, 18). As outlined above, Dredd is an essential component of the Imd signaling pathway. In Dredd mutant animals or Dredd knockdown cells, neither Imd nor Relish are cleaved and AMP genes are not induced. Moreover, forced expression of Dredd in S2 cells causes cleavage of Imd and Relish even in the absence of immune stimulation, and immunoprecipitated Dredd was able to cleave Relish in vitro (7, 10). Altogether, these results suggest that both Imd and Relish are direct substrates for Dredd-mediated cleavage.

In this report, we aim to verify that Imd and Relish are direct Dredd substrates and provide further insights on the mechanisms of Dredd activity. We show that Dredd is the only caspase that can trigger Imd and Relish cleavage, and that partially purified recombinant Dredd can cleave Imd and Relish in vitro. Moreover, Dredd activity does not require interdomain cleavage and this autoprocessing is not observed during immune activation. Additionally, we show that baculovirus p35, a potent suicide inhibitor of some caspases, does not inhibit Dredd activity and does not block the Imd signal transduction pathway, contrary to previous reports.

EXPERIMENTAL PROCEDURES

Cell Culture—Drosophila S2* cells were maintained in Schneider’s Drosophila media (Invitrogen) supplemented with 10% fetal bovine serum (Valley Biomedical), 1% GlutaMAX (Invitrogen), and 0.2% penicillin-streptomycin (Invitrogen) at 27 °C. D.mel-2 cells were maintained in serum-free SFX-insect medium supplemented with 20 mM glutamine was inoculated with Nc in certain apoptotic contexts (15, 16). Dredd with Nc in certain apoptotic contexts (15, 16). Dredd

Immunoblotting and Immunoprecipitation—Following stimulation with PGN or induction with CuSO₄, cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.6, 10% glycerol, 1% Triton X-100, 150 mM sodium chloride, 25 mM β-glycerophosphate, 2 mM EDTA, 1 mM DTT, 1 mM sodium orthovanadate, 1 μM protease inhibitor mixture). For whole cell lysate analysis, 50 μg of total protein was separated by SDS-PAGE and immunoblotted with anti-Imd (7) or anti-Rel-C (8) antibodies. Images were captured using LAS4000 imager following ECL detection and quantified with ImageJ. For Fig. 1B, the percent of Relish cleavage was calculated based on the ratio of cleaved product relative to the density of total (cleaved and full-length) Relish in each lane. For detection of caspases, 200–500 μg of total protein extract was used for immunoprecipitation with anti-V5 antibody (Sigma) or anti-T7 antibody (Millipore) and subjected to immunoblotting.

Purification of Recombinant Proteins—Stably transfected S2* cells expressing FLAG-DreddWT-V5, FLAG-DreddCA-V5, FLAG-RelishWT, and FLAG-RelishD545A were plated at a density 0.5 × 10⁶ cells/ml and incubated for 20 h. Cells were treated with 500 μM copper sulfate to induce expression of recombinant proteins for 4 h. Cells were harvested, washed with 1× PBS, and lysed at 80 °C. Cells were lysed in 50 ml of lysis buffer without glycerol per 1 liter of culture and centrifuged at 20,000 × g for 30 min at 4 °C. Supernatants were collected and loaded onto 0.5 ml of anti-FLAG-agarose affinity media (Sigma). For experiments in Figs. 3 and 5, RNA was isolated using TRIzol reagent (Invitrogen) and cDNA was synthesized using Mutagenesis Kit (Agilent Technologies).

Transfection—Drosophila S2* cells and D.mel-2 cells were transfected using the calcium phosphate method as described previously (20). For experiments in Figs. 3 and 5C, cells were transiently transfected. For all other experiments, stable cell lines were established with either hygromycin (24 units/ml) or G418 (800 μg/ml) selection.

PGN Stimulation—For PGN stimulation, cells were plated at a density of 0.5 × 10⁶ cells/ml and grown for 16 h, and then treated with 1 mM 20-hydroxyecdysone (Sigma) for an additional 24 h. Expression of V5-tagged Dredd was then induced with 5 (Fig. 4) or 500 μM (Fig. 5, A and B) copper sulfate for 4 h. Cells were stimulated with 1 μg/ml of PGN extracted from Escherichia coli strain 1106 (ATCC 35581).
reaction mixtures were TCA precipitated with 10 μg of BSA carrier and analyzed by immunoblotting.

Caspase Assays Using Purified Recombinant Dredd—Caspase assays were performed as previously described with modifications (21). 200 ng of wild type and catalytically inactive Dredd were assayed in 100 μl of caspase assay buffer (100 mM HEPES, pH 7.4, 10% sucrose, 50 mM sodium chloride, 0.5 mM EDTA, 0.1% CHAPS, 10 mM DTT, and 1 mM PMSF) or sodium citrate buffer (0 to 1.4M sodium citrate, pH 7.4, 10 mM DTT, and 1 mM PMSF) at 30 °C for 2–4 h using 10 μM fluorogenic peptide substrates, Ac-IETD-AMC (caspase-8 substrate), Ac-LEHD-AMC (caspase-9 substrate), and Ac-YVAD-AMC (caspase-1 substrate).

RNA Analysis—Total RNA was extracted and analyzed by Northern blotting or real-time RT-PCR as previously described (20).

Fly Stocks—Yp1-Gal4 (3), GMR-rpr (22), and UAS-p35 (23) were previously described. Yp1-Gal4/+ and UAS-p35/+ flies were generated by crossing homozygous transgenic lines to w1118, whereas the Yp1-Gal4/UAS-p35 animals were generated with a cross between these same lines.

RESULTS

Dredd Is Unique Among Drosophila Caspases in Processing Both Imd and Relish—Previously, we showed that over-expression of Dredd in S2* cells was sufficient to drive cleavage of both Imd and Relish in the absence of immune stimulation (7, 10). To more fully characterize the caspases potentially involved in these immune signaling events, we established and analyzed stable cell lines over-expressing each of the seven Drosophila caspases, using the copper-inducible metallothionein promoter. The proteases examined included three apical caspases,
Dredd, Nc, and Dream, and four effector caspases, Ice, Dcp1, Damm, and Decay, as well as a catalytically inactivate variant of Dredd. Of the caspases assayed, only wild type Dredd promoted the cleavage of Relish or Imd (Fig. 1). The degree of Imd cleavage observed in the cell line expressing wild type Dredd was similar (or higher) to that detected in the parental S2* cells stimulated with peptidoglycan. On the other hand, the degree of Relish cleavage, caused by Dredd over-expression, was lower but still robust and reproducible. Of note, Decay expressing cells displayed an atypical Relish processing event, even without copper induction, suggesting a possible Decay cleavage site in Relish. However, this cleavage has no known link to Imd pathway activation. Overall, these data suggest that only Dredd, among the 7 Drosophila caspases, is involved in Imd and Relish cleavage.

Purified Recombinant Dredd Cleaves Imd and Relish Proteins in Vitro—To test whether Imd and Relish are substrates for Dredd, we purified recombinant wild type and catalytically inactive FLAG-Dredd-V5 from stably transfected S2* cells, using anti-FLAG M2 affinity gel capture and FLAG peptide elution. The resulting Dredd preparations were partially pure, with a major full-length Dredd band, around 52 kDa, at least two Dredd cleavage products, and 3 to 6 copurifying bands visible by Coomassie Blue staining (supplemental Fig. S1). A small fraction of wild type Dredd underwent spontaneous auto-processing to generate several fragments, which are not observed with the catalytically inactive Dredd (supplemental Figs. S1 and S2).

These Dredd preparations were next used to monitor proteolytic activity with fluorogenic peptide substrates. Using the fluorogenic “caspase-8” substrate Ac-IETD-AMC, along with increasing concentrations of the kosmotropic salt sodium citrate, wild type but not the catalytically inactive Dredd exhibited strong activity. This activity was dependent on sodium citrate, reaching maximal levels with 1.2 M sodium citrate, suggesting that Dredd requires multimerization for full activity (Fig. 2A).

On the other hand, the presence of kosmotropic salt appeared to inhibit autoprocessing; that is, less autoprocessed Dredd fragments were detected in reactions with the highest sodium citrate concentrations (Fig. 2B, white arrowheads). Supplemental Fig. S2 shows that the production of these Dredd fragments required catalytically active Dredd, arguing that they are all autocleavage products. Dredd also cleaved the “caspase-9” substrate Ac-LEHD-AMC, with similar activity, but showed little activity for the “caspase-1” substrate Ac-YVAD-AMC (Fig. 2C).

Caspase inhibitors such as Z-VAD-fmk, Ac-IETD-fmk, and Ac-LEHD-fmk were all capable of inhibiting Dredd in a dose-dependent manner, using the Ac-IETD-AMC assay, although Ac-IETD-fmk was most potent at concentrations below 5 μM (Fig. 2D).
To generate protein substrates, His-tagged Imd and FLAG-tagged Relish were similarly expressed in stably transfected D.mel2 or S2* cells and purified by nickel capture and imidazole elution or anti-FLAG M2 affinity gel and FLAG-peptide elution, respectively. These proteins were incubated with wild type or catalytically inactive recombinant Dredd in caspase assay buffer, and assayed by immunoblotting. Wild type Dredd cleaved recombinant Imd and Relish proteins in vitro, whereas catalytically inactive Dredd did not. Moreover, this cleavage occurred at the established cleavage site for each substrate, as the uncleavable mutants of Imd (D30A) and Relish (D545A) were not processed by Dredd in vitro (Fig. 2E). Together, these data show that Imd and Relish are both direct substrates for Dredd.

**Figure 3. Uncleavable Dredd mutants still support Imd and Relish cleavage.** A, a schematic representation of the Dredd and the mutant constructs analyzed here. Candidate sites (aspartate residues) for autoproteolysis, based on the homology between Drosophila Dredd and human caspase-8, were mutated to alanine or asparagine. B–D, different mutants of Dredd were expressed from the copper-inducible metallothionein promoter in stably transfected S2* cells, and Dredd autoprocessing (top) as well as, Imd (middle), and Relish cleavage (lower) were monitored by immunoprecipitation (IP) and immunoblotting (IB) for Dredd-V5, or straight immunoblotting for Imd, Relish. The putative small subunit, p10, was never detected in these assays. Dotted line in panel B contrasts different mobilities of p30 fragments between D2A and wild type Dredd. Black arrowhead, full-length Dredd, Imd, or Relish; white arrowhead, cleaved forms of Dredd, Imd, or Relish. Results shown are representative of at least three independent assays.

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**Autoprocessing of Dredd**—In the experiments above, we observed a few processing intermediates from wild type Dredd. A fragment just above the 31-kDa standard, which we named p30, was most prominent in Coomassie staining and V5-immunoblotting after purification (supplemental Fig. S1). A similar fragment was also observed in extracts from stably transfected cells and the appearance of this fragment also required Dredd activity (Figs. 1 and 3). Based on its size, we hypothesize that this fragment arises from a cleavage between the prodomain and catalytic domains (see Fig. 3A). To identify possible processing sites in Dredd, two aspartate residues in the linker region between the prodomain and catalytic domain were changed to alanine (D2A), but this mutant was still processed to p30 (although the migration of this p30 band is slightly altered) and was still able to cleave Imd and Relish when over-expressed (Fig. 3B, note the dotted line for comparison). This suggests that processing to generate p30 either does not occur at either of these two aspartate residues (260 and 271) or can occur at a nearby alternative sites when these two aspartates are mutated.

We similarly analyzed the role of inter-domain cleavage, which would separate the putative large and small catalytic subunits of Dredd (Fig. 3A). In immunoblotting either the cell-based over-expression assays or the in vitro cleavage reaction, the small subunit (p10) was almost never detected (Figs. 1 and 2B). In the bottom of Fig. 2B, a very faint signal for a possible p10-like product can be observed, but only in the 0.2 M sodium citrate reaction, where the peptide substrate were not cleaved (marked by an asterisk). Thus, the detection of p10 does not correlate with active Dredd. To examine interdomain cleavage further, three aspartate residues in the region separating the putative large and small subunits were substituted with alanine (D3A). This mutant, as well as the D5A mutant lacking all five candidate processing site aspartates, showed greatly reduced Imd or Relish cleavage (Fig. 3B). Single aspartate to alanine mutants were analyzed to deconvolute the D3A mutant, and only the D436A mutant failed to cleave Imd and Relish (Fig. 3C), suggesting that processing at this site may be required for Dredd function. However, the Asp → Ala substitution is a dramatic change that may effect overall protein structure, so a more conservative D436N substitution was similarly tested. Surprisingly, D436N sub-
stitution still autoprocessed and cleaved both Imd and Relish similar to wild type (Fig. 3D). We also substituted the interdomain region with the tobacco etch virus protease recognition site and cotransfected this version of Dredd with increasing concentrations of a tobacco etch virus protease expressing plasmid (supplemental Fig. S3). Although this construct generated a clearly detectable p10 fragment in a tobacco etch virus-dependent manner, it did not drive the cleavage of Imd or Relish. These results suggest that interdomain cleavage of Dredd, separating the large and small subunits, is neither necessary nor sufficient to cause its activation and cleavage of downstream targets.

To monitor the processing of Dredd in a more immunologically relevant situation, we induced Dredd expression at a moderate level with low copper sulfate (5 μM) in stably transfected S2* cells. This level of Dredd expression did not trigger the cleavage of Imd or Relish. These cells were then stimulated with 1 μg/ml of PGN and lysates were prepared at various time points up to 30 min poststimulation. The full-length and putative cleavage products of Dredd were immunoprecipitated with the C-terminal V5 tag and then immunoblotted. Although robust immune activation and signaling was observed, as monitored by the PGN-induced cleavage of both Imd and Relish, no processing of Dredd was observed; the full-length protein remained intact for the duration of the experiment and the protein banding pattern was indistinguishable from catalytically inactive Dredd (Fig. 4). All together, these results suggest that interdomain cleavage of Dredd, both to remove the prodomain and/or to separate the large and small subunits, is not involved in the immune activation of Dredd nor a critical part of the Imd signaling cascade.

**Baculovirus p35 Does Not Inhibit Dredd-mediated Cleavage of Imd and Relish**—Baculovirus p35 is a highly specific inhibitor of caspases that prevents apoptotic cell death in mammalian and *Drosophila* cells (23, 24). p35 itself is a substrate of caspases and remains covalently bound to the catalytic cysteine to inhibit activity (25). Two previous reports have concluded that p35 inhibits Imd signaling, presumably by inhibiting Dredd (3, 26). However, other reports have claimed that p35 specifically inhibits effector caspases, like Ice, but not apical caspases like Nc (27, 28). The inhibitory activity of p35 on Dredd has not been carefully examined. To address this apparent contradiction, we first expressed p35 from the copper-inducible metallothionein promoter in stably transfected S2* cells. These cells were stimulated with 1 μg/ml of PGN for 10 min, and whole cell lysates were prepared and analyzed by immunoblotting for Imd and Relish cleavage (Fig. 5A and supplemental Fig. S4A). Surprisingly, expression of p35 did not affect the immune-induced cleavage of Imd or Relish. Also, the PGN-induced expression of *Diptericin*, *Attacin A*, and *Cecropin A1* was unaffected by p35 in these cell lines (Fig. 5B).

As p35 is cleaved by the caspases it targets, we next determined which of the seven *Drosophila* caspases are capable of this processing. For this assay, p35 and each of the *Drosophila* caspases were transiently transfected into S2* cells. As expected, p35 was efficiently processed in Dcp1 and Ice overexpressing cells, but no cleavage product was observed in the Dredd overexpressing cells (Fig. 5C, top panel). Dcp1 and Ice were even found in the same immunoprecipitated complex with p35 in immunoprecipitation-immunoblot experiments (supplemental Fig. S4B). Of note, Damm but not Decay, which are two other effector caspases from *Drosophila*, cleaved p35 but to a lesser extent (Fig. 5C). In vitro caspase assays showed that recombinant Dredd did not cleave purified p35, whereas commercial human Caspase-3 readily cleaved p35 (Fig. 5D). These data strongly argue that p35 is not a substrate for Dredd in vitro and, accordingly, it does not inhibit cleavage of Imd or Relish in cells.

Based on these in vitro results, we next re-examined the effects of p35 on Imd signaling in adult animals. We ectopically expressed p35 in the fat bodies of adult female flies using a UAS-p35 transgene and the Yp1-Gal4 driver. Importantly, this assay included three controls genotypes: *w^1118* is the genetic background for these strains; the UAS-p35 transgene crossed into the *w^1118* background; and *Yp1-Gal4* crossed into the *w^1118* background. These last two control genotypes were omitted from earlier studies (3, 26). These four genotypes were left unmanipulated, or were injected with either sterile PBS or live *E. coli*, to maximally stimulate the Imd pathway. Six hours later,
RNA was isolated and the induction of Diptericin was quantified by real-time quantitative RT-PCR. In comparison to the w1118 strain, it is immediately clear that the amplitude of Diptericin gene expression is dramatically muted in the Yp1-Gal4 strain, although the fold-induction remains comparable (309-fold in w1118 versus 176-fold in Yp1-Gal4). Thus, it is critical to compare the Yp1-Gal4/UAS-p35 animals to this driver-alone control; in this comparison, it is clear that p35 expression has no negative effect on Diptericin induction. In fact, the levels are increased over the control Yp1-Gal4 strain (474-fold increase in E. coli infected flies compared in uninfected flies). Quantitative RT-PCR analysis, in the lower panel of Fig. 6A, showed that p35 is expressed, as expected, in the Yp1-Gal4/UAS-p35 animals. To ensure that the UAS-p35 transgene functions properly, i.e. that is capable of inhibiting caspase-mediated cell death (22), the same UAS-p35 transgene was expressed in the developing eye imaginal discs with the GMR-Gal4 driver along with the apoptotic inducer reaper (rpr) (Fig. 6B). Expression of p35 efficiently suppressed the reaper-induced small and rough eye phenotype, as previously reported (22). Taken together, these results demonstrate that p35 is not an inhibitor of Dredd and does not interfere with immune signaling and AMP gene expression through the Imd pathway.

DISCUSSION

In this study, we have focused on two key steps in the Drosophila Imd signaling pathway. Previous work has shown that both Imd and Relish are cleaved in a caspase-dependent
manner, and genetic studies suggested that the caspase-8-like Dredd was responsible for both of these proteolytic events. Here, we present compelling evidence that Dredd, uniquely among the 7 Drosophila caspases, is able to directly cleave both of these proteins. One caveat remains with the in vitro biochemical assays presented here, as all proteins were expressed and purified from Drosophila S2* cells, rather than a heterologous system. Thus, our protein preparations may contain co-purifying/interacting factors that contribute to these cleavage reactions. Nonetheless, the cell-based over-expression results combined with the biochemical assays strongly argue that Dredd is uniquely and directly cleaving both Imd and Relish.

Caspases often autocleave, both to remove their prodomain and to generate mature p20 and p10 catalytic subunits (29). However, the putative small catalytic subunit (p10) of Dredd was almost never detected in our assays with either over-expressed S2* cell-based assays or purified Dredd in vitro. In contrast, mammalian caspase-8 requires interdomain cleavage for its activation during apoptosis (30). Perhaps, more similar to our results with Dredd, activation of caspase-8 does not require interdomain cleavage in certain non-apoptotic roles, such as LPS-induced B-lymphocyte proliferation or when complexed with FLIP<sub>L</sub> (31, 32). Caspase-9 is another example of initiator caspase that does not require interdomain cleavage for its activation and apoptotic function (33–35). Like Caspase-9, unprocessed Dredd is likely to function as part of a multimeric complex in the Imd signaling pathway.

Although the mature p10 domain was not detected in the purified and active Dredd samples, we repeatedly observed several p30-related fragments, possibly indicating multiple cleavage sites between the prodomain and the catalytic domain. Two candidate cleavage site aspartates were substituted with alanine (D2A), however, these failed to prevent generation of p30-related fragments, although the processed fragment in the D2A mutant displayed a slightly faster migration on SDS-PAGE, suggesting alternative cleavage sites. Regardless of the exact location(s) of cleavage to generate the p30-related fragments, this processing does not appear to be critical for Dredd activation during immune signaling, and may even function to inactivate Dredd. Instead, the Death effector domain prodomain is likely critical to form a higher order multimeric complex of Dredd along with Fadd, through Death effector domain homotypic interactions, and this complex likely also includes Imd, which interacts with Fadd through the

**FIGURE 6.** Baculovirus p35 protein does not block the Imd pathway signaling in vivo. A, p35 was ectopically expressed in female fat bodies using the Gal4/UAS system and the Yp1-Gal4 driver. p35 expressing animals and control genotypes (w<sup>1118</sup>, UAS-p35 alone, and Yp1-Gal4 alone) were injected with either PBS or live E. coli 1106, whereas uninfected flies were included as a control. After 6 h of immune challenge, real-time RT-PCR analysis was performed to quantify Dipteracin induction (top panel) as well as p35 expression (bottom panel). Rp49 was used as reference for normalizing expression. The level of Dipteracin induction was notably lower in the Yp1-Gal4 alone animals compared with the wild type strain. Expression of p35, in the UAS-p35/Yp1-Gal4 animals, actually increased Dipteracin expression, relative to the driver-only control. Error bars indicate SD, from two biological replicates. B, the same UAS-p35 transgene was expressed in the developing eye using GMR-Gal4. Expression of p35 strongly suppressed reaper-induced apoptosis in the eye, as expected. Images shown are representative from multiple number of animals for each group.
Imd and Relish Are Dredd Substrates

homotypic Death Domain interface. The quaternary structure of this complex is currently unclear, as well it is not known if this complex is preformed, and activated by PGN stimulation, or if it forms upon immune activation through association with the receptor PGRP-LC.

As shown in Figs. 1 and 2E, we observed that only a small fraction of Imd undergoes cleavage following immune stimulation, or forced expression of Dredd in cells, or with purified Dredd in vitro. One explanation of these observations is that Imd has a relatively low affinity for active site of Dredd. Alternatively, it is also plausible that only a fraction of Imd has a cleavage site that is available to Dredd. For example, it was recently shown that RIP1 and RIP3, mammalian counterparts of Imd, form a functional amyloidal signaling complex in vitro and in vivo (36). If Imd similarly forms amyloidal complexes, many of these molecules could have their Dredd cleavage sites obscured within the fibrils. A third possibility is that some of the Imd protein is found in compartments of the cell not available to Dredd. Consistent with this, a large proportion of Imd is found in nucleus (37).

On the other hand, most of the Relish expressed in S2* cells was rapidly cleaved following PGN stimulation, suggesting that this process is very efficient. However, forced expression of Dredd was able to cleave only a fraction of Relish. This implies that forced expression of Dredd lacks some unknown factor(s) or modification(s) that are required for efficient cleavage of Relish. One possible modification is polyubiquitination of Dredd and/or Imd. We recently showed that K63-polyubiquitination of both Imd and Dredd are important for Imd signal transduction and these polyubiquitin chains are suggested to form molecular platforms for downstream signaling events (7, 38). We hypothesize that these K63-polyubiquitin chains may enhance Dredd-mediated Relish cleavage, and they are lacking when Dredd is over-expressed. Further studies are required to investigate this hypothesis.

Baculovirus p35 protein inhibits many caspases in vitro and apoptosis in vivo (39, 40). Co-crystal structure of p35 and caspase-8 revealed that the aspartate of p35 in the reactive loop forms a thioester with the active cysteine of caspase-8 and undergoes a dramatic conformational change that prevents hydrolysis of this thioester (25). Expression of p35 inhibits NC-dependent apoptosis by antagonizing effector caspases Ices and Dcp1, but it does not inhibit NC itself (27, 28). Two earlier reports concluded that p35 inhibits Imd signaling, thus suggesting that p35 inhibits Dredd (3, 26). On the other hand, here we present biochemical and genetic evidence demonstrating that p35 is not a Dredd inhibitor and accordingly does not inhibit Imd and Relish cleavage, nor AMP gene induction. Importantly, our genetic assays include critical control genotypes, which were absent from earlier studies. The Yp1-Gal4 driver alone is particularly critical, as it shows a marked reduction in AMP expression compared with other wild type strains.

In this study, we show that Dredd is the only Drosophila caspase that can promote Imd and Relish cleavage, and that Imd and Relish are direct substrates of Dredd. It will be interesting to learn if it is the same Dredd complex that is responsible for cleaving Imd and Relish, or if Dredd forms distinct complexes to target each of these substrates.

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