Insect Biochemistry and Molecular Biology 108 (2019) 16–23

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Regulation of the *Drosophila* Imd pathway by signaling amyloids

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ARTICLE INFO

Keywords:
Drosophila immunity
Imd signaling
Functional amyloid

ABSTRACT

Fruit flies elicit effective defense responses against numerous microbes. The responses against Gram-negative bacteria are mediated by the Imd pathway, an evolutionarily conserved NF-κB pathway recognizing *meso*-diaminopimelic acid (DAP)-type peptidoglycan from bacterial cell walls. Several reviews already provide a detailed view of ligand recognition and signal transduction during Imd signaling, but the formation and regulation of the signaling complex immediately downstream of the peptidoglycan-sensing receptors is still elusive. In this review, we focus on the formation of the Imd amyloidal signaling center and post-translational modifications in the assembly and disassembly of the Imd signaling complex.

1. Formation of an amyloidal signaling complex

1.1. Ligand binding and receptor interactions

In *Drosophila,* signaling through the Imd pathway is triggered by DAP-type peptidoglycan (DAP-PGN), which is commonly found in Gram-negative bacteria, but also in some Gram-positive species, such as *Bacillus* spp. and *Listeria* (Lemaitre and Hoffmann, 2007; Stenbak et al., 2004). DAP-PGN is released from the bacterial cell wall during cell division and bacterial cell death, both in polymeric and monomeric forms (Dworkin, 2014). PGN is sensed by the *Drosophila* immune system through direct binding to receptors known as Peptidoglycan Recognition Proteins, or PGRPs, which are encoded by 13 distinct genes, some of which encode multiple splice isoforms (Dziarski, 2004; Royet and Dziarski, 2007). Of these, PGRP-LC and PGRP-LE are specific receptors to DAP-PGN (Kaneko et al., 2006; Takehana et al., 2004) and mediate Imd pathway signaling (Choe et al., 2002; Gottar et al., 2002; Rämet et al., 2002; Takehana et al., 2002). PGRP-LC is a transmembrane receptor found at the cell surface. Heterodimers of PGRP-LC splice-isoforms PGRP-LCx and -LCa (referred to as PGRP-LC-PA and –LC-PB, respectively, in Flybase) recognize monomeric DAP-PGN, while PGRP-LCx alone is sufficient to recognize long, polymeric PGN (Chang et al., 2006; Kaneko et al., 2004; Lim et al., 2006; Werner et al., 2003). The role of the third signaling isoform, PGRP-LCy (PGRP-LC-PC in Flybase) is still elusive. Biophysical studies have established that PGRP-LCa and PGRP-LCx dimerize upon binding to monomeric DAP-PGN. On the other hand, polymeric DAP-PGN binding is assumed to cluster PGRP-LCx, which is sterically prevented from forming homo-multimers, to drive signaling. PGRP-LE is a cytosolic receptor that detects DAP-PGN delivered into the cytoplasm and multimerizes upon ligand binding (Lim et al., 2006). PGRP-LE is important for sensing DAP-PGN released from intracellular bacteria, such as *Listeria* (Yano et al., 2008) or from live, extracellular bacteria, which release monomeric DAP-PGNs that can be transported into the cytosol (Neyen et al., 2016; Park and Uehara, 2008). The SLC46A family transporter CG8046 is involved in transporting monomeric DAP-PGNs into the cytosol for recognition by PGRP-LE, especially in the immune responsive insect renal organ, the Malpighian tubules (Paik et al., 2017).

Both PGRP-LC and PGRP-LE belong to the class of long PGRPs and contain a conserved C-terminal PGRP-domain as well as an extended N-terminal region. These N-termini are critical for downstream signaling but contain no predicted domains or obvious signaling motifs (Choe et al., 2005; Kaneko et al., 2006). Therefore it remained, for many years, very unclear how ligand binding to the PGRP domain mechanistically activates signal transduction through these N-terminal domains.

Abbreviations: AMP, antimicrobial peptide; Atg1, Autophagy-related 1; DAI, DNA-dependent activator of IFN-regulatory factors also known as ZBP-1; DAP, *meso*-diaminopimelic acid; DED, Death effector domain; Dredd, death-related ced-3/Nedd2-like protein; FADD, Fas-associated protein with Death Domain; Diap2, Drosophila inhibitor of apoptosis 2; IκB, inhibitor of κB; IKK, IκB kinase; Imd, immune deficiency; NF-κB, Nuclear factor κB; PGN, peptidoglycan; PGRP, peptidoglycan recognition protein; RHIM, RIP homotypic interaction motif; RIPK, receptor-interacting protein kinase; SMOC, supramolecular organizing center; Tab2, Tak1-associated binding protein 2; Tak1, TGF-β activated kinase 1; TRIF, TIR-domain-containing adapter-inducing interferon-β

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https://doi.org/10.1016/j.ibmb.2019.03.003
Received 22 January 2019; Received in revised form 19 February 2019; Accepted 5 March 2019
Available online 09 March 2019

Interestingly, PGRP-LC expression is relatively low (Neyen et al., 2012) and overexpression induces Imd signaling (Choe et al., 2005). In fact, just a small increase in the amount of PGRP-LCx is enough to promote signal transduction (Rus et al., 2013). Additionally, overexpression of ectodomain-deleted PGRP-LC is sufficient to drive robust Imd signaling (Choe et al., 2005). These results suggest a "proximity hypothesis," whereby clustering of PGRP-LC or PGRP-LE, caused by ligand binding or overexpression, causes the N-terminal regions to cluster into a higher order structure sufficient to trigger downstream signaling events. The low abundance of PGRP-LC on the plasma membrane, and the lack of specific and sensitive antibodies, have limited our ability to test this proximity hypothesis with endogenous proteins. However, expression of PGRP-LC is naturally dependent on the insect steroid hormone ecdysone, which is best known for its role regulating development and metamorphosis (Rus et al., 2013). Ecdysone levels peak during pupation when flies have low exposure to environmental bacteria, and this ecdysone peak drives increased levels of *PGRP-LC* during metamorphosis. This elevated expression of *PGRP-LC* is sufficient to induce Imd signaling, including antimicrobial peptide (AMP) gene expression as well as the NF-κB–dependent induction of *Atg1* and autophagy (Nandy et al., 2018), further supporting the N-termini proximity hypothesis as the trigger of downstream signaling events.

1.2. RHIM motifs and the formation of the amyloid core

The PGRP-LC and PGRP-LE N-termini are largely dissimilar from each other except for a short stretch of sequence with weak homology to the mammalian RIP Homotypic Interaction Motifs (RHIMs) (Fig. 1) (Kaneko et al., 2006). This sequence motif is referred to as a cryptic RHIM (cRHIM) and is essential for signaling by both receptors, but the function of these cRHIMs remained unclear for many years. In 2012, Li et al. reported that the mammalian RHIMs fold into cross-beta sheet conformations and form functional amyloid fibrils. Moreover, this amyloidal RHIM structure was causally linked to RIPK1/3-dependent necroptotic signaling in mammalian cells (Li et al., 2012). The insect cRHIMs include the characteristic patterns of serines, asparagines, and hydrophobic residues that promote beta sheet formation (Fig. 1), as well as the more conserved four amino acid core that is characteristic to all RHIMs (Chan et al., 2015; Kleino et al., 2017). However, the Drosophila cRHIMs lack a Gln that is highly conserved in the core of the mammalian RHIMs. Mammalian RHIMs have been reported in multiple proteins linked to necroptotic signaling, including RIPK1, RIPK3, DAI, and TRIF (Kaiser et al., 2008; Kaiser and Offermann, 2005; Rebsamen et al., 2009; Sun et al., 2002). In flies, cRHIMs have been identified in the Imd signaling proteins PGRP-LC, PGRP-LE, the receptor proximal adaptor protein Imd, as well as Relish - the key NF-κB transcription factor in this pathway (Chan et al., 2015; Dushay et al., 1996). The cRHIMs of PGRP-LC, –LE and Imd are critical for Imd signaling, but the cRHIM in the N-terminus of Relish has not been functionally or biochemically characterized. In addition, a putative cRHIM is present in one of the PGRP-LA isoforms, PGRP-LA-PD, which is expressed in epithelia, and contributes to triggering the local antimicrobial response through the Imd pathway, possibly via a cRHIM interaction (Gendrin et al., 2013). Like the Relish cRHIM, the PGRP-LA-PD cRHIM requires more in-depth analysis. On the other hand, the cRHIMs in PGRP-LC, PGRP-LE, and Imd were recently characterized biophysically and functionally. In particular, all these proteins and their cRHIMs were found to form amyloids, *in vitro* and in cells, and amyloid formation was found to be required for Imd signaling. In particular, blocking cRHIM amyloid activity, by mutation or with small molecular inhibitors, interfered with Imd signaling (Kaneko et al., 2006; Kleino et al., 2017).

How does the proximity of RHIMs result in the formation of an amyloidal signaling complex? Structural details of *Drosophila* cRHIMs are still elusive, but the crystal structure of the conserved, four-amino acid core motif of the mammalian necrosome complex was recently solved. The core motifs of RIPK1 and RIPK3, IQIG and VQVG, respectively, stack into a heteroamyloid, where RIPK1 and RIPK3 RHIMs alternate, forming cross-beta sheets. Two of these sheets then bind together through hydrophobic interactions, tightly packing the hydrophobic isoleucine and valine residues inside the structure (Mompeán et al., 2018). Residues pointing out of the hydrophobic core, such as the conserved Gln of the core motif, and the Asn residues flanking the RHIM core, stabilize the amyloid structure through hydrogen bonding along the fibril axis, as in a ladder (Mompeán et al., 2018). *Drosophila* cRHIMs lack the conserved Gln residue in the cRHIM core. However, the pattern of hydrophobic residues in cRHIM cores of PGRP-LC, PGRP-LE, and Imd is similar to mammalian RHIMs. Furthermore, the cRHIMs can functionally substitute the mammalian motif in chimeric molecules (Kleino et al., 2017), suggesting that the *Drosophila* cRHIMs might form a similar tightly packed hydrophobic core as mammalian RHIMs. Current experimental evidence suggests that PGRP-LC and PGRP-LE cRHIMs form the amyloidal nucleus that further promotes interaction with Imd, and subsequent conversion of the Imd cRHIM into amyloidal fibrils. Whether this requires an additional cellular translocation or membrane targeting of Imd is still unknown. It is also unclear whether the *Drosophila* cRHIM fibrils are stabilized through the Asn residues, which are found flanking the three *Drosophila* cRHIM cores, as the mammalian RIPK1-RIPK3 fibrils, and whether His residues within the cRHIM core of PGRP-LE and Imd can form amyloid structure-stabilizing hydrogen bonds as Gln does in the mammalian RHIM amyloid. It is also currently unknown if the amyloid core is formed around a short PGRP-LC or PGRP-LE homoamyloid nucleus, or as a receptor-Imd heteroamyloid fibril, similar to the RIPK1/RIPK3 alternating beta-sheet structures. Further structural analysis is required to resolve these questions.

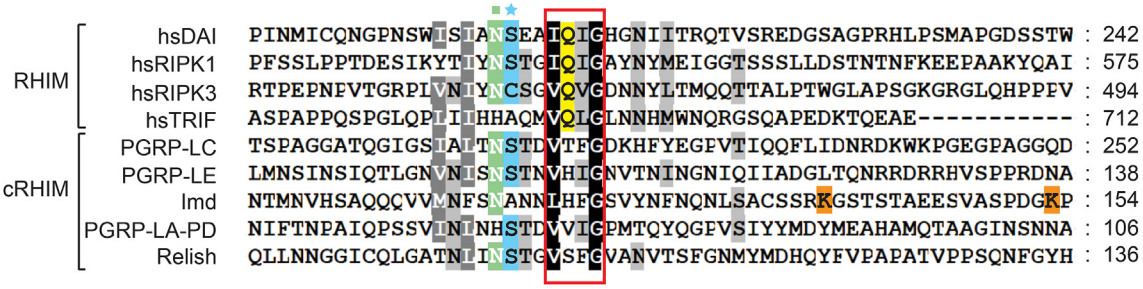

Fig. 1. Comparison of mammalian RHIM sequences and *D. melanogaster* cRHIMs. The four core amino acids are boxed with red. The Gln (Q) residues that are highly conserved in mammalian RHIMs and stabilize the RIP amyloid fibril through hydrogen bonding, but are missing in *Drosophila* cRHIMs, are highlighted with yellow. Ser (S) and Cys (C) that stabilize RIPK1/RIPK3 heteroamyloid through Cys-Ser ladders, are highlighted with cyan and a star. Asn (N) that forms stabilizing hydrogen bonds in RIPK1/RIPK3 amyloid is highlighted in light green and a square. Lysines (K137 and K153) that are K63-ubiquitinated in Imd are highlighted with orange. Shading represents conservation according to Blosum62 scoring matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

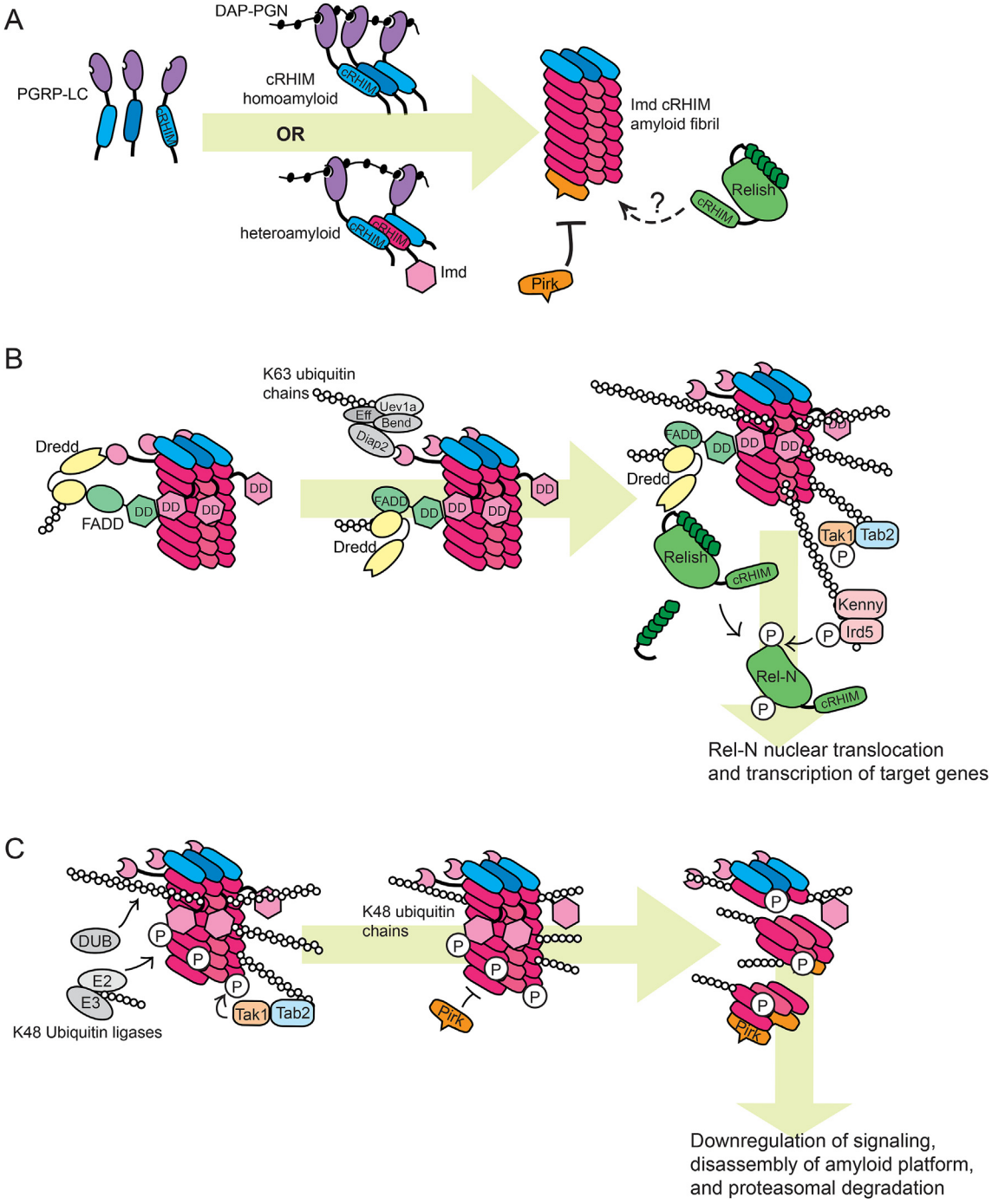

Fig. 2. Model of the assembly and disassembly of the amyloidal Imd SMOC. A) PGN binding to the receptor PGRP-LC triggers cRHIM-mediated amyloidal aggregation. A nucleus formed either by the receptor alone (homoamyloid), or receptor and Imd cRHIMs (heteroamyloid) seeds the formation of an Imd homo-amyloid fibril. Whether Relish also integrates the fibril through its putative cRHIM is unknown. The negative regulator Pirk inhibits fibril formation possibly by capping the nascent fibrils. Similar amyloid SMOC formation is also hypothesized when PGRP-LE binds PGN ligands within the cytosol. **B)** Imd cRHIM amyloid functions as the signaling platform, with the N-terminus and the Death domain (DD) facing out of the fibrillar structure (lighter shade of pink). FADD brings the caspase Dredd to the fibril via a DD-mediated interaction. Dredd cleaves Imd and creates a docking site for Diap2, which together with E2 ligases conjugates Imd with K63-ubiquitin chains. K63-ubiquitin chains further recruit the Tab2-Tak1 complex as well as the IKK complex (Ird5 and Kenny) into the SMOC. IKK activation leads to phosphorylation while Dredd may cleave Relish within this amyloidal SMOC. Cleaved Relish then translocates to the nucleus to drive transcription of target genes. **C)** Imd phosphorylation by Tak1 drives removal of K63-ubiquitin chains, and the subsequent linkage with K48-ubiquitin chains to Imd by yet unknown DUBs and ubiquitin ligases. Together with Pirk, these phosphorylation and K48-ubiquitination events promote disassembly of the amyloid platform and proteasomal degradation of Imd, downregulating Imd signaling. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2. Signal transduction downstream of Imd: amyloid and beyond

Overall, our model posits that the Imd amyloid fibril, which is triggered by PGRP-LC or PGRP-LE proto-amyloid seeds, creates a large platform to support the events required for further signal transduction events (Fig. 2A). Such large signaling platforms have been noted before, especially in the context of innate immune signaling, and are referred to as supramolecular organizing centers (SMOCs) (Kagan et al., 2014). However, without detailed structural knowledge about the Imd cRHIM it is not possible to definitively know which amino acids participate in amyloid fibril formation and how an Imd amyloid SMOC might be organized. Nonetheless, reasonable hypotheses can be proposed building on structural information from mammalian systems as well as the genetic and molecular analyses of the Imd signaling pathway. It is plausible that the cRHIM core motif of Imd, LHFG (amino acids 118–121), forms a hydrophobic fibril core similar to IQIG and VQVG motifs from RIPK1 and RIPK3, respectively. But how does this fibril promote Imd signal transduction?

In addition to the cRHIM, activation of signaling by Imd requires both its N- and C-termini to be accessible for other protein-protein interactions, suggesting that both termini extend away from the core amyloid fibril. The C-terminus of Imd harbors a death domain, which mediates the interaction with the death domain of *Drosophila* FADD (Georgel et al., 2001; Leulier et al., 2002; Naitza et al., 2002). FADD in turn recruits the Caspase 8 homolog Dredd (Leulier et al., 2000). Dredd is an initiator caspase with a long N-terminal prodomain containing two adjacent Death Effector Domains (DEDs). Activation of Dredd does not require autoproteolytic cleavage (Kim et al., 2014). Instead, the prodomain of Dredd interacts with FADD as well as the ubiquitin E3 ligase Diap2, possibly through DED1, which promotes Dredd K63-polyubiquitination and activation (Meinander et al., 2012). Once activated, Dredd plays a dual role in the Imd pathway activation, cleaving both Imd and the transcription factor Relish (Kim et al., 2014). Dredd cleaves Imd after Asp 30 to create a neo-N-terminus with a docking site for Diap2 (Paquette et al., 2010). Diap2, together with the E2 ligases Effete (Ubc5), Bendless (Ubc13), and Uev1a conjugate the Imd Lys residues K137 and K153 with K63-polyubiquitin chains (Chen et al., 2017). This cleavage and K63-ubiquitination of Imd occurs rapidly after immune stimulus. In cultured cells, cleaved Imd can be detected within a minute of DAP-PGN exposure, followed by a peak in K63-ubiquitinated Imd around 20–40 min post-induction. K63-polyubiquitin chains attract downstream signaling proteins to the signaling complex (Chen et al., 2017; Paquette et al., 2010). Intriguingly, K137 resides is in proximity of the amyloid forming cRHIM of Imd. Although we do not know yet how far the amyloidal region in Imd spans in its three dimensional conformation, we hypothesize that both K137 and K153 should be accessible to ubiquitin conjugation, and therefore either on the outer surface of the fibril or extending away from the fibril (Fig. 2B).

In addition to ubiquitination, Imd is also post-translationally modified by the MAPK3 Tak1 (Chen et al., 2017). Tak1 is recruited to the signaling complex by its interacting partner and adaptor protein Tab2 (Kleino et al., 2005). Tab2 contains an N-terminal CUE domain and a C-terminal ZnF, which have been associated with ubiquitin binding in other organisms, the ZnF showing specificity to K63 ubiquitin chains in mammals (Bagola et al., 2013; Kulathu et al., 2009). Our current hypothesis posits that Tab2 binds the K63 ubiquitin chains linked to Imd (and possibly to Dredd), bringing the Tak1 complex into the larger Imd fibril context. Tak1 directly phosphorylates Imd, and this phosphorylation down-regulates Imd signaling by promoting Imd degradation (Chen et al., 2017). However, Tak1 also phosphorylates the IKK complex, which in *Drosophila* consists of the regulatory subunit Kenny (IKKγ or NEMO homolog), and the kinase Ird5 (IKKα/β homolog) (Rutschmann et al., 2000; Silverman et al., 2000). The details of how Kenny is recruited to the signaling complex are still unclear, but the recruitment likely also involves K63 ubiquitin chains, as Kenny has a predicted K63-binding motif, a NZF domain, in its very C-terminus. Interestingly, Kenny was recently also reported to be a target of M1-linked (linear) ubiquitination (Aalto et al., 2018). The molecular function of these M1 chains is not yet clear, although it was argued that the M1-generating enzyme (LUBEL in *Drosophila*) is required for the defense against oral, but not septic, infection (Aalto et al., 2018). In any case, similar to the mammalian NF-κB systems, Tak1-mediated phosphorylation of Ird5 likely activates the *Drosophila* IKK complex, which then phosphorylates and further activates the transcription factor Relish. Relish activation requires both its cleavage and phosphorylation, mediated by Dredd and IKK respectively (Erturk-Hasdemir et al., 2009; Silverman et al., 2000; Stoven et al., 2003). It is not known if the *Drosophila* IKK complex has other targets, beyond Relish.

It is not clear how or if Relish is recruited to the Imd fibrillar signaling complex. One possibility is that a putative N-terminal cRHIM in Relish might be involved in the SMOC by physically connecting Relish to the Imd fibrils (Chan et al., 2015). Relish recruitment into the amyloid fibril would allow proximity with Dredd, which is necessary for its cleavage. However, how a cleaved Relish-N might then escape the Imd amyloid to translocate to the nucleus is completely opaque. It is possible that phosphorylation of Relish might play a role in its disassembly from the amyloid. Alternatively, despite the proposed cRHIM in its N-terminus, Relish-N may never come into contact with the Imd amyloid fibril. Instead, the phosphorylation and cleavage of Relish may occur in another subcellular location after Dredd and IKK are released from Imd SMOC. This is a fundamental question for current research – do all cytosolic signaling events occur within the context of the supramolecular complex formed on the Imd amyloid fibril, or are non-amyloid components released from this fibril once activated to perform their critical functions elsewhere in the cell? Better cell biological tools will need to be developed to address this question.

3. Downregulation and disassembly of the PGRP-LC-Imd signaling complex

Activation of innate immune response has a significant cost on fitness and reproduction (Bischoff et al., 2006; McKean et al., 2008; Zerofsky et al., 2005). Not surprisingly, Imd signaling is subject to multiple levels of regulation, providing mechanisms to return to homeostasis after an acute response (Myllymaki et al., 2014). For example, the Imd pathway is downregulated at steps from DAP-PGN recognition through signal transduction. At the level of PGN recognition, secreted PGRPs, PGRP-SB1, -SB2, -SC1, -SC2, and LB, are all active amidases that can digest polymeric DAP-PGN into short fragments unable to trigger immune response, thereby limiting the availability of initial stimuli (Kurata, 2014). This mechanism is especially important in the gut, where the epithelial cells are continuously exposed to microbes and their metabolites. Consistent with this notion, PGRP-SC1/2, and PGRP-LB are expressed in the gut (Bischoff et al., 2006; Charroux et al., 2018; Zaidman-Rémy et al., 2006).

At the level of receptor complex formation, transmembrane PGRPs PGRP-LF, and the recently discovered alternatively spliced regulatory isoforms of PGRP-LC (rPGRP-LC), appear to act as decoy receptors or decoy interaction partners and thereby inhibit signaling (Maillet et al., 2008; Neyen et al., 2016; Persson et al., 2007). PGRP-LF contains two extracellular, adjacent PGRP domains, a transmembrane domain, and a very short cytoplasmic part that lacks a cRHIM and therefore cannot support signal transduction. The function of the PGRP domains of PGRP-LF in the suppression Imd signaling is not completely clear. PGRP-LF was reported to bind PGN, which suggested that it might compete with PGRP-LC for ligand binding (Persson et al., 2007). However, the crystal structure of PGRP-LF PGRP domains does not support this, as the PGN binding cleft in both domains is obstructed and does not permit PGN binding. Instead, PGRP-LF strongly interacted with the ectodomain of TCT-bound PGRP-LCx, suggesting that PGRP-LF could limit Imd signal transduction by competing with PGRP-LCa for ligand-dependent receptor dimerization (Basbous et al., 2011). How the

two PGRP domains of PGRP-LF function together (*i.e.* whether they bind each other when inactive), and whether PGRP-LF can bind PGRP-LCx bound to polymeric PGN is not yet clear.

In addition to the three signaling PGRP-LC isoforms mentioned in section 1.1, the *PGRP-LC* locus also encodes three alternatively spliced regulatory isoforms (rPGRP-LCs). The regulatory isoforms harbor PGRP domains corresponding to the signaling isoforms, a transmembrane domain, and a short cRHIM-deficient cytoplasmic region that does not support signaling (Neyen et al., 2016). Instead, the cytoplasmic part of the rPGRP-LC isoforms harbors a PHD-type (Cys4-His-Cys3) zinc finger motif. The PHD motif structurally resembles the phosphoinositide-binding FYVE domain, and has been shown to bind lipids (DiNitto et al., 2003). The PHD of rPGRP-LCs targets the protein to membrane microdomains, but is also involved in its interaction with the negative regulator Pirk as well as the E3 ubiquitin ligase Diap2 (Neyen et al., 2016). The role of these interactions in the regulatory function of rPGRP-LC is still unclear, although the current evidence suggests that Pirk may act as a sorting adaptor in recycling and regulating PGRP-LC and rPGRP-LC (Lhocine et al., 2008; Neyen et al., 2016). rPGRP-LC can sequester PGRP-LC away from the plasma membrane and target it into an inactive membrane compartment for ubiquitination and subsequent degradation. In addition, rPGRP-LC competes with PGRP-LC for PGN binding and receptor-receptor interactions by forming signaling deficient receptor complexes (Neyen et al., 2016).

3.1. Pirk in amyloid regulation

Pirk is probably the best characterized of the Imd pathway negative regulators, yet our understanding of the many aspects of its inhibitory action is far from complete. Pirk was identified as a direct interaction partner of the PGRP-LC and PGRP-LE cRHIM motifs, as well as Imd, and it was shown to negatively regulate both the humoral immune response and tolerance to gut microbiota (Aggarwal et al., 2008; Kallio et al., 2005; Kleino et al., 2008; Lhocine et al., 2008). Further, *pirk* transcription is regulated by Relish, and Pirk thus acts as a negative feedback regulator (Aggarwal et al., 2008; Kleino et al., 2008). During microbial challenge, *pirk* expression peaks at 1 h post-induction, providing only a limited time for Imd-mediated responses before Pirk starts tuning it down.

Pirk interacts with both PGRP-LC/LE and Imd but not as part of a receptor/adapter complex, and was therefore hypothesized to act by preventing the receptor-Imd interaction through steric hindrance (Aggarwal et al., 2008; Kleino et al., 2008). Pirk has also been suggested to participate in clearing the receptor from the plasma membrane and targeting it into vesicular compartments, possibly lysosomes for degradation (Lhocine et al., 2008), or through endosomal recycling in concert with rPGRP-LC as a response to PGN from dead bacteria (Neyen et al., 2016). To date, similar recycling or sequestration mechanisms have not been reported for PGRP-LE, which may indicate a different role for Pirk relative to these two receptors.

Pirk is a 197 amino acid protein with no recognizable domains or motifs. The central portion of Pirk (amino acids 51–136) includes a repetitive region while the very C-terminal region includes a computationally predicted RHIM (Kajava et al., 2014). Unlike the central repetitive region, the putative RHIM is not evolutionarily conserved in other insects, arguing against it being functionally important. Studies with deletion constructs have demonstrated that both the central repetitive region and the C-terminal region, including the putative RHIM, are sufficient to block the Imd signaling. $Pirk_{51\text{-}136}$ showed stronger binding to Imd, while the C-terminal end seemed to prefer PGRP-LC as a binding partner (Kleino et al., 2008). The biological significance of these two regions having independent inhibitory activity is not yet clear.

Recombinant Pirk forms amyloid-like aggregates *in vitro*. In addition, these aggregates can be detected in lysates of Pirk overexpressing cells (unpublished data). However, it is still unclear if endogenous Pirk forms amyloid fibrils in cells, or if these aggregates are important in the inhibitory activity of Pirk. The amyloidal properties of Pirk are intriguing as we now know that the core of the Imd signaling complex is also amyloidal and formed around the nucleus of PGRP-LC or PGRP-LE cRHIMs propagating Imd fibrillation. *In vitro*, recombinant Pirk can block the propagation of Imd fibrils. In addition, overexpression of Pirk dissolves, or at least reduces the size of amyloid fibrils formed by Imd, PGRP-LCx, or PGRP-LE (Kleino et al., 2017). Whether Pirk integrates into the fibrils to destabilize them or prevents further fibrillation by capping the nascent fibrils is still unknown.

3.2. Ubiquitination and ubiquitin editing

Toxicity of various amyloid species and amyloidal proteins is somewhat controversial, but accumulating evidence suggests that both amyloid fibrils and oligomers are harmful to cells (Marshall et al., 2014). However, the surprising prevalence of functional amyloids throughout the kingdoms of life suggests that the cells also have sophisticated and efficient mechanisms to control amyloidal protein aggregation by sequestering them to specific compartments and/or controlling their assembly and disassembly. Of known functional amyloids, human Pmel17 fibrils are sequestered into melanosomes as tightly packed, organized structures that are visible by electron microscopy (Fowler et al., 2007). Bacterial amyloids contributing to pilus formation are kept monomeric by regulatory proteins until secreted, after which they quickly organize into mature amyloid fibrils (Deshmukh et al., 2018). Amyloidal translational regulators, such as CPEB proteins regulating long-term memory, or the fungal meiosis regulator Rim4, can switch between the oligomeric (amyloidal) and monomeric forms by becoming post-translationally modified by sumoylation or phosphorylation (Carpenter et al., 2018; Drisaldi et al., 2015). It is unclear if the mammalian RHIM fibrils ever disassemble, since necroptosis signaling results in cell death that hardly leaves room for sophisticated mechanisms of amyloid clearance. However, Imd signaling is efficiently downregulated and does not result in cell death, at least not in the physiological context, suggesting that the Imd amyloidal signaling complex is disassembled in an orderly manner. The mechanisms of Imd amyloid disassembly are unclear, but autophagy and proteasomal degradation are likely candidates. Autophagy has been associated with PGRP-LE-dependent clearance of Listeria (Yano et al., 2008), starvation-induced production of antimicrobial peptides (Wu et al., 2007), degradation of the IKK complex (Tusco et al., 2017), and most recently with Relish- and PGRP-LC-dependent salivary gland degradation (Nandy et al., 2018), but no evidence is currently available supporting autophagy as a means for removing amyloidal Imd signaling complexes. However, proteasomal degradation has been reported to play a role in the downregulation of Imd signaling (Chen et al., 2017; Khush et al., 2002).

The adaptor protein Imd undergoes several post-translational modifications in the succession of signal transduction. Imd is K63-ubiquitinated within minutes after DAP-PGN exposure, which further promotes the signal transduction by recruiting other signaling components to the complex. One of these components is Tak1, which phosphorylates not only the IKK complex, but also Imd (Chen et al., 2017). While the Imd phosphorylation site(s) is still unknown, this phosphorylation step is dependent on the Diap2-mediated K63-ubiquitination of Imd and seems critical for subsequent modification of these ubiquitin chains. While a strong K63-ubiquitination signal can be detected in S2* cells already 2–5 min after PGN exposure, decreasing to undetectable levels in 40 min post-induction, the level of K48-ubiquitinated Imd peaks later, at 15–20 min (Chen et al., 2017). This ubiquitin editing is Tak1 dependent and is probably due to de-ubiquitination and subsequent addition of K48-linked ubiquitin chains to Imd. Both the enzymes de-ubiquitinating Imd, and the K48-linking E3 ligase remain unknown, although CYLD and Usp 36 are possible candidate DUBs (Thevenon et al., 2009; Tsichritzis et al., 2007). Proteasome inhibition

results in the accumulation of K48-ubiquitinated Imd, underlining the importance of proteasome in the termination of Imd signaling. The most parsimonious explanation to connect these findings with the amyloidal Imd SMOC, discussed above, would suggest that Imd is rapidly activated by K63-ubiquitinated in the amyloid SMOC (Fig. 2B), and then is quickly subject to regulatory modifications. Pirk controls amyloid fibril stability while DUBs and a yet-to-be identified E3s drive amyloid disassembly, ubiquitin editing and Imd turnover via the proteasome. Given the fitness costs of immune activation and the possible toxicity of amyloid fibrils this tight regulation is likely essential for supporting a robust, transient and effective immune response.

4. Final thoughts

Since its discovery in 1995, Imd has proved to be a fascinating molecule (Lemaitre et al., 1995). Initial analyses indicated that the Death Domain of Imd was most similar to that in RIPK1 (Georgel et al., 2001). Although Imd does not include a kinase, this connection to RIPK1 has now been expanded to also include the cRHIM in Imd (as well as in PGRP-LC and PGRP-LE), and the amyloid fibril forming activities of these motifs. The *Drosophila* cRHIMs can substitute for their mammalian counterpart in the context of RIPK3 chimeras, and form amyloids *in vitro* and in cells. However, these *Drosophila* amyloids are likely to have some important differences in their physical properties, compared to the mammalian RIPK1/3 fibrils. This is highlighted by differences in their primary sequences. The cRHIM core motifs of PGRP-LC, PGRP-LE, and Imd lack the conserved Gln residue that in mammalian proteins contributes to stabilizing the fibrils through hydrogen bonding. More importantly, the *Drosophila* cRHIMs lack a Cys residue proximal to the core motif, which is present in RIPK3 (Cys455, highlighted in cyan in Fig. 1). Cys455 stabilizes RIPK1/RIPK3 heteroamyloid through hydrogen bonding with Ser 536 of RIPK1, and promotes the formation of highly stable RIPK3 homoamyloid through disulfide bonding (Mompeán et al., 2018). In *Drosophila* PGRP-LC and PGRP-LE, this position includes a Ser, which potentially could make stabilizing hydrogen bonds. However, Imd includes an Ala at this position, possibly indicating less stable fibril conformation through the length of the Imd amyloid SMOC. This hypothesis requires further biophysical study. Regardless of their absolute stability, the *Drosophila* cRHIM fibrils are uniquely sensitive to disassembly and/or termination by Pirk, a property which tracks with the 11 amino acid cRHIM sequence (Kleino et al., 2017). Interestingly, the inhibitory regions of Pirk (51–136 and 137–197 amino acids) are both predicted to consist mainly of alternating beta sheets and loops, which may give us a clue of how Pirk could interfere with receptor-Imd amyloid fibril formation. A similar sheet-loop-sheet structure is present in bacterial proteins forming functional amyloids. For example, CsgA, which is the major subunit of the amyloidal Curli pilus in *E. coli*, contains five beta sheet-forming repeats separated by loops, while a pilus protein from *Pseudomonas*, FapC, contains three amyloidogenic repeats with extended loops (Deshmukh et al., 2018; Rasmussen et al., 2019). These bacterial amyloids do not contain RHIMs, but the amino acid composition of the amyloidal regions resembles that of known RHIMs and seems to parallel what is predicted for the secondary structure of Pirk. These similarities suggest Pirk may assemble into nascent Imd fibril, terminating further fibrillary growth, possibly destabilizing the fibrils, and facilitating K48-ubiquitination, disassembly, and proteasomal degradation (Fig. 2C). Future studies will examine these similarities and differences amongst the RHIM amyloids and the role of Pirk in regulating their activity and structure.

Funding

This work was supported by NIH grant AI060025 to N.S. A.K. is an AIAS-COFUND Fellow (FP7 Marie Curie Actions – People) supported by the European Union's Seventh Framework Program for research, technological development and demonstration under grant agreement no 609033 and by Aarhus University Research Foundation (AUFF).

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