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Pirk Is a Negative Regulator of the Drosophila Imd Pathway

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NF-κB transcription factors are involved in evolutionarily conserved signaling pathways controlling multiple cellular processes including apoptosis and immune and inflammatory responses. Immune response of the fruit fly Drosophila melanogaster to Gram-negative bacteria is primarily mediated via the Imd (immune deficiency) pathway, which closely resembles the mammalian TNF signaling pathway. Instead of cytokines, the main outcome of Imd signaling is the production of antimicrobial peptides. The pathway activity is delicately regulated. Although many of the Imd pathway components are known, the mechanisms of negative regulation are more elusive. In this study we report that a previously uncharacterized gene, pirk, is highly induced upon Gram-negative bacterial infection in Drosophila in vitro and in vivo. pirk encodes a cytoplasmic protein that coimmunoprecipitates with Imd and the cytoplasmic tail of peptidoglycan recognition protein LC (PGRP-LC). RNA interference-mediated down-regulation of Pirk caused Imd pathway hyperactivation upon infection with Gram-negative bacteria, while overexpression of pirk reduced the Imd pathway response both in vitro and in vivo. Furthermore, pirk-overexpressing flies were more susceptible to Gram-negative bacterial infection than wild-type flies. We conclude that Pirk is a negative regulator of the Imd pathway.

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3 Abbreviations used in this paper: PGRP, peptidoglycan recognition protein; AttB, Attacin B; CecB, Cecropin B; DptB, Diptericin B; Iap2, inhibitor of apoptosis protein 2; Imd, immune deficiency; luc, luciferase construct; Pirk, poor Imd response upon knock-in; Puc, Puckered; RNAi, RNA interference; qRT-PCR, quantitative RT-PCR; Tak1, TGF-β-activated kinase; ToM, TurandotM.
Materials and Methods

Genome-wide analysis of mRNA levels in S2 cells by oligonucleotide microarrays
dsRNA treatments, Imd pathway induction, total RNA extractions, and oligonucleotide microarrays were performed as described previously (20).

Cell culture and transfections

S2 cells, which are Drosophila hemocyte-like cells, were maintained in Schneider’s insect cell culture medium (Sigma-Aldrich) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 25°C. All transfections were performed with FuGENE transfection reagent (Roche Applied Science) according to the manufacturer’s instructions.

dsRNA treatments and luciferase assays
dsRNA treatments and luciferase assays for monitoring Imd and Toll pathway activities were performed as described earlier (20, 17, 29). To monitor Jak/STAT pathway activity, the cells were transfected with TotM (Turan-dotm)-luciferase (-luc) reporter plasmid and constitutively active hop-scotch, which activates Jak/STAT signaling. Cell viability was assessed using Actin5C-β-galactosidase reporter. Transfections were performed on 24-well plates and 1 μg of dsRNA was used for each dsRNA treatment. The pirk overexpression construct was created by cloning CG15678 (from S2 cell cDNA to pMT/V5/His-A vector (Invitrogen Life Technologies). Deletion mutants PirkΔM1–K51, PirkΔK52–V136, and PirkΔV137–I197 were created by cloning nucleotides 1–153, 154–408, and 409–591, respectively, from pirk cDNA to pMT/V5/His-A vector.

Drosophila stocks

CS64-GAL4 driver flies were obtained from Prof. B. Lemaitre (Centre de Génétique, Centre National de la Recherche Scientifique, Gif-Sur-Yvette, France) and CG15678 RNAi flies were from Prof. R. Ueda (National Institute of Genetics, Mishima, Shizuoka, Japan). The pirk cDNA was ligated with a V5 epitope tag and cloned into a pfuUST plasmid (30). Flies overexpressing pirk were generated by microinjecting pfuUST-pirk-V5 constructs to w1118 background in the Umea Fly and Worm Transgene Facility, Umeå, Sweden. Independent UAS-pirk-V5 transgenic fly lines used in experiments were referred to as UAS-pirk221, UAS-pirk231, UAS-pirk422, and UAS-pirk721, having the insertion in the third, X, third, and second chromosomes, respectively.

Infection and RNA extraction from flies

Flies were infected by pricking them with a tungsten needle dipped into a concentrated culture of Enterobacter cloacae 0, 1, 4, or 8 h before RNA extraction. Triplicates of five flies (three males and two females) were snap frozen in dry ice and homogenized in TRIZol reagent (Invitrogen Life Technologies), and the total RNAs were extracted according to the manufacturer’s instructions. For survival assay, 50 flies of each line were infected as described above and their survival was monitored for 48 h at 25°C.

Protein extraction from flies and Western blotting

To extract total proteins, 10 flies of each line were anesthetized, snap frozen in dry ice and homogenized in 80 μl of lysis buffer (10 mM Tris (pH 8.0), 140 mM NaCl, 1% Triton X-100, and Complete mini protease inhibitor cocktail (Roche Applied Science)). Fly extracts were incubated on ice for 45 min, centrifuged at 16,000 × g for 15 min, and the protein concentration of the cleared lysates was measured using a BCA protein assay kit (Pierce). Forty micrograms of each lysate was electrophoresed in NuPAGE 10% Bis-Tris gel (Invitrogen Life Technologies), blotted on a nitrocellulose membrane, and detected by Western blotting using mouse anti-V5 tag. Expression level of the cleared lysates was measured using oligonucleotide microarrays. One hundred percent (100%) indicates the maximum induction of each gene. B. pirk induction is Relish-dependent in vitro. S2 cells were treated with either control (GFP) or Relish dsRNA 48 h before Imd pathway activation with heat-killed E. coli. Total RNAs were extracted at the 4-h time point and used in oligonucleotide microarrays. C. pirk induction is Relish-dependent in vivo. Wild-type (Canton S) and RelishΔ20 flies were infected by E. cloacae and the expression level of pirk was quantitated by qRT-PCR at 0, 1, 4, and 8 h after infection. Error bars represent the SD; n = 3.

Quantitative real-time PCR

Quantitative RT-PCR (qRT-PCR) was performed by either a LightCycler (Roche) or an ABI7000 (Applied Biosystems) instrument using the Quant iTec SYBR Green RT-PCR kit (Qiagen) according to the manufacturer’s instructions. Results were analyzed with the LightCycler version 3.5 software or ABI 7000 System SDS software version 1.2.3. Primers contained the following sequences: Actin5C (Act5C), 5′-CCAAAGAAATGGTGCTGCT CTGG-3′ (forward) and 5′-AGAACGATACCGGTGGTACG-3′ (reverse); ArtB, 5′-CAGTTCACACACAGGACC-3′ (forward) and 5′-CT CCTGCTGGAAGACATCC-3′ (reverse); CecB, 5′-TTGTGGCACTCA GACGGCGA-3′ (reverse); and 5′-TCCTGG-3′ (forward) and 5′-TCCAGGAGACCTTGATGTA-3′ (reverse); Dpht, 5′-GACTGGCTTTGCTCCT-3′ and 5′-CCTGAAGGTATACACT CC-3′ (reverse); pirk, 5′-CCATGACGAGGTCTCACAC-3′ (forward) and 5′-TGCGTCCAGGATGATC-3′ (reverse); and puc, 5′-GAGCGCGA GCCGTGAGTC-3′ (forward) and 5′-GCCGTGATGATGACCTGC-3′ (reverse).

Confocal microscopy

S2 cells were seeded onto 24-well plates and transfected with 0.1 μg of a modified pMT/GFP/V5/His plasmid (a gift from D. Klein) expressing Pirk as C-terminal GFP fusion protein. Pirk expression was induced 24 h later by adding CuSO4 to a final concentration of 100 μM. Forty-eight hours after induction the cells were fixed with 3.7% formaldehyde and 5% sucrose for 20 min. The coverslips were washed three times with PBS and mounted to objective glasses with Vectashield mounting medium for fluorescence with 4′,6-diamidino-2-phenylindole (Vector Laboratories).

Coinmunoprecipitation and Western blotting

S2 cells were seeded onto 6-well plates and transfected with 0.5 μg of C-terminally V5-tagged full-length pirk construct or deletion construct in pMT/V5/His-A vector (Invitrogen Life Technologies) and 0.5 μg of myc-tagged imd, Iap2, and Taki or the cytoplasmic tail of PGRP-L2M1-V291 in the same vector of which the V5 tag was replaced by myc tag. Expression of the tagged proteins was induced 24 h post-transfection by adding CuSO4 to a final concentration of 250 μM. Cells were harvested 48 h later and lysed in 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 50 mM
NaCl, 0.5% sodium deoxycholate, 20 mM NaF, 1% Nonidet-P40, 10% glycerol, 100 μg/ml PMSF, and Complete mini protease inhibitor cocktail (Roche Applied Science)). Lysate volume corresponding to 1 mg of total protein for each sample (as measured by BCA protein assay kit; Pierce) was first precleared by adding 25 μl of a 1:1 suspension of protein G-Sepharose (GE Healthcare) in lysis buffer and incubating the mixture for 50 min with rotation at 4°C. The resulting supernatants were transferred to fresh tubes. Twenty-five microliters of protein G-beads were added to 1 g of anti-c-Myc rabbit IgG Ab. The samples were incubated overnight with rotation at 4°C and then washed with PBS containing PMSF and protease inhibitors for 4 × 10 min. Immunoprecipitates were separated from the beads by adding 25 μl of 2 × SDS loading buffer, vortexing, and incubating at 95°C for 5 min, then electrophoresing in NuPAGE 10% Bis-Tris gel (Invitrogen Life Technologies), and blotting on nitrocellulose membrane. Proteins were detected by 1/5000 diluted mouse anti-V5 or rabbit anti-c-Myc primary Ab and by goat anti-mouse or anti-rabbit Ab HRP conjugates (Invitrogen Life Technologies), respectively, and visualized by ECL Plus Western blotting detection system (GE Healthcare).

Statistical analysis

Statistical analysis of results was conducted using one-way ANOVA and, for survival experiments, logrank analysis. p < 0.05 was considered significant.

Results

Pirk is induced upon Gram-negative bacterial infection in vivo and in vitro in Relish-dependent manner

Antimicrobial response of Drosophila is delicately regulated and includes a precise temporal control. Drosophila Imd pathway transcription factor Relish contains both NF-kB and IκB homologous parts and is activated when the C-terminal IκB part is cleaved. We hypothesized that inhibition of the Imd signaling is mediated by a negative feedback loop. In theory, a putative negative regulator

**FIGURE 2.** Pirk is a negative regulator of the Drosophila Imd pathway in vitro. A, Knocking down pirk by RNAi increases Imd pathway-mediated AttA response at 4, 8 and 24 h compared with GFP controls. S2 cells were transfected with AttA-luc reporter plasmid and treated with dsRNAs, and the Imd pathway was induced 48 h later by heat-killed E. coli. GFP dsRNA was used as a negative control and Relish dsRNA as a positive control. B–D, Imd pathway mediated induction of antimicrobial peptide genes is increased in pirk RNAi knock-down cells. S2 cells were treated with pirk dsRNA 48 h before adding heat-killed E. coli, and total RNAs were extracted at 0, 1, 4, and 24 h after induction. The levels of pirk (B), AttB (C), and DptB (D) transcripts were analyzed by qRT-PCR and normalized to Actin-5C transcript levels. E, In vitro overexpression of pirk suppresses Imd pathway activation in S2 cells. S2 cells were transfected either with an empty vector or a pirk overexpression construct together with AttA-luc reporter. Imd pathway was activated by heat-killed E. coli. F and G, Pirk does not affect Toll pathway activity but decreases Jak/STAT-mediated TotM response. S2 cells were transfected with Drosomycin-luc (F) or TotM-luc (G) reporter plasmids for Toll and Jak/STAT pathway, respectively, and treated with dsRNAs. Luciferase activity was measured 72 h posttransfection. Drosophila MyD88 (dmMyD88) dsRNA was used as a positive control for Toll pathway and STAT dsRNA for Jak/STAT pathway. hop, hopscotch.
should be induced upon microbial challenge via the Imd pathway. Previously, we have identified all genes that are induced upon Gram-negative bacterial challenge via the Imd pathway in S2 cells (29). In this study, RNAi targeting a gene of unknown function, CG15678, enhanced Imd pathway activity in vitro. To further examine the role and function of this gene, which we call pirk, we first monitored the expression kinetics of the genes induced upon heat-killed Escherichia coli treatment. According to microarray analysis, 11 genes were expressed ≥5-fold in S2 cells (20). Fig. 1 shows the relative induction profiles of these genes. Most of them were steadily induced and reached their maximum induction at 24 h after bacterial challenge. Antimicrobial peptide gene CecB (Cecropin B) was an exception, reaching its maximum at 4 h. However, only pirk was rapidly induced, reaching its maximum already at 1 h postinfection and then decreasing.

To examine whether this induction is Relish-dependent, we treated S2 cells with either control (GFP) or Relish dsRNA 48 h before inducing the Imd pathway activation by E. coli and analyzed pirk expression levels at 4 h by oligonucleotide microarrays. Fig. 1B shows that pirk expression levels were abolished after

Relish RNAi. To confirm this in vivo, we infected wild-type (Canton S) and Relish null mutant (Relish<sup>220</sup>) flies by septic infection with E. cloacae and monitored pirk expression levels by qRT-PCR at 0, 1, 4, and 8 h after bacterial challenge. pirk was highly induced in Canton S but not detected in Relish<sup>220</sup> flies (Fig. 1C). These results indicate that pirk expression is Relish-dependent both in vitro and in vivo.
Pirk specifically suppresses Imd pathway activity in vitro

To study the role of Pirk in Imd pathway regulation, we used an Attacin A-luc reporter-based assay in S2 cells treated either with control (GFP dsRNA as negative and Relish dsRNA as positive control) or pirk dsRNA, together with heat-killed E. coli to activate the Imd response. Fig. 2A shows AttA-luc activity representing Imd pathway activation measured at 1, 4, 8, and 24 h after induction with heat-killed E. coli. AttA-luc activity in pirk dsRNA-treated cells was increased at all time points studied. The results were similar with Cecropin A1-luc reporter (data not shown). We also monitored expression levels of Imd pathway-dependent antimicrobial peptide genes in S2 cells treated either with control (GFP) or pirk dsRNA by quantitative RT-PCR. Again, the Imd pathway was activated by adding heat-killed E. coli to the cells. As expected, pirk expression in pirk RNAi knock-down cells was strongly decreased, indicating the effectiveness of the dsRNA treatment (Fig. 2B). Fig. 2, C and D show the relative expression levels of AttB (Attacin B) and DptB (Dipterycin B), which were significantly higher in pirk dsRNA-treated cells compared with the GFP dsRNA-treated controls. In conclusion, pirk RNAI increases Imd pathway activation in vitro.

Next, we tested whether in vitro overexpression of pirk affects Imd pathway activity. S2 cells were transfected with luciferase reporter construct together with either empty or pirk overexpressing pMT vector, and the Imd pathway was activated by adding heat-killed E. coli to the cells. Relative AttA-luc reporter activities are shown in Fig. 2E. pirk overexpression reduced Imd pathway activation by >70%, indicating that Pirk suppresses Imd signaling in S2 cells.

To study whether Pirk action is Imd pathway specific, we tested the effect of pirk RNAI on activation of the Toll (Fig. 2F) and Jak/STAT (Fig. 2G) pathways. For analyzing the Toll pathway, S2 cells were transfected with Drosomycin-luciferase (Drs-luc) reporter and, to activate the pathway, constitutively active Toll10B together with GFP (negative control), cMyd88 (positive control), or pirk dsRNA. For Jak/STAT pathway, transfections were conducted with TotM-luc reporter together with GFP (negative control), STAT (positive control), and pirk dsRNAs, and the pathway was activated by overexpressing the Janus kinase hopscotch. pirk RNAI did not affect the activity of the Toll pathway. Similar results were obtained by activating the Toll pathway with an active form of the Spätzle ligand (data not shown). However, pirk RNAI significantly reduced Jak/STAT pathway activity. These results indicate that Pirk is not a general inhibitor of signaling cascades in S2 cells.

Pirk suppresses Imd pathway activity in vivo

To examine the effect of Pirk on Imd pathway activation in vivo, we first monitored Imd pathway-mediated, antimicrobial peptide gene expression levels in pirk in vivo in RNAI flies by qRT-PCR. Two independent UAS-RNAI lines, CG15678 R1 and -R2 were crossed to C564-GAL4 driver (31) to target pirk-RNAI construct expression to hemocytes and fat body, and Imd pathway activity in a week-old offspring was induced by septic injury with E. cloacae by septic injury. AttB, CecB, and DptB expression levels were analyzed by qRT-PCR at 0 and 4 h postinfection (Fig. 4A and B, respectively). AttB, CecB, and DptB were highly expressed at 4 h postinfection in control flies. The expression levels were significantly lower in pirk-overexpressing flies compared with uninduced controls. This indicates that Pirk suppresses the Imd pathway activity in vivo.

Pirk is a cytoplasmic protein interacting with Imd and cytoplasmic part of PGRP-LC

Pirk is a 197-aa protein with no recognizable signal sequence or previously characterized domain structure. No Pirk homologues have been described from other species, but a tblastn search of the assembled genomic sequences on the Flybase web site (flybase.bio.indiana.edu) identified likely orthologs in 18 sequenced holometabolous insect genomes. They all share a central conserved domain, but the N-terminal and C-terminal regions differ between different insect orders. The central domain, which we call the Pirk domain, shows a weakly conserved repetitive structure with three copies of an 18-aa repeat and two copies of a possibly related 12-aa repeat, referred to as A1–3 and B1–2, respectively (Fig. 5). In all cases the open reading frames were uninterrupted by introns. Additional exons may exist, but cDNA and expressed sequence tag sequences support the absence of introns in Drosophila melanogaster, Aedes aegypti, and Bombyx mori. The four different B. mori sequences probably include allelic variants of two to three paralogous genes. Curiously, we found no homologue in the Anopheles genome.

Pirk has no recognizable signal sequence and is thus expected to be an intracellular protein. To test this, we visualized the cellular localization of an expressed GFP-Pirk fusion protein using confocal microscopy. GFP-Pirk was detected as green dots in the cytoplasm outside the blue 4′,6-diamidino-2-phenylindole (DAPI)-stained nucleus, as shown in Fig. 6A. Treatment of GFP-Pirk-expressing S2 cells with E. coli did not affect Pirk’s localization (data not shown). Pirk was also detected in cell extracts but not in the cell culture medium by Western blotting (data not shown). These results imply that Pirk is a cytoplasmic protein.

To study the mechanism of Pirk-mediated inhibition of Imd signaling, we conducted epistasis analysis using the active form of Relish. S2 cells were transfected with Rel ΔS29–45 and CuSO4-inducible pirk construct together with AttA-luc reporter construct to see whether overexpression of Pirk affected Relish-induced Imd pathway activity (AttA-luc expression). No effect was detected, indicating that Pirk is located upstream of Relish in the Imd signaling cascade (Fig. 6B).

The Drosophila Imd pathway also triggers an immediate response via JNK pathway, because the JNK pathway branches from the Imd signaling cascade at the level of Tak1 (22, 41). To find out whether Pirk suppresses the Imd pathway activity upstream of Tak1, we analyzed the effect of pirk RNAI on JNK pathway activity. This was performed by measuring the expression levels of a JNK pathway target gene, puc (Puckered), by qRT-PCR. pirk RNAI increased puc expression in S2 cells (Fig. 6C), indicating that Pirk negatively regulates the JNK pathway-mediated response as well. This suggests that Pirk suppresses the Imd pathway activity at the level of or upstream of Tak1.

To determine the mechanism for Pirk-mediated Imd pathway suppression, we coimmunoprecipitated V5-tagged Pirk with the
Myc-tagged Imd pathway components Iap2, Tak1, Imd, or the cytoplasmic tail of PGRP-LC (LC(M1–V203)) in S2 cells. As shown in Fig. 6D, Pirk coimmunoprecipitated with Imd and the cytoplasmic part of PGRP-LC. In addition, a faint band was visible in Iap2 samples, but not in Tak1 samples. These results imply that Pirk is likely to interact with Imd and the cytoplasmic tail of PGRP-LC and either directly or indirectly with Iap2, but not with Tak1. Therefore, it is likely that Pirk suppresses Drosophila Imd signaling by interacting directly with Imd and the cytoplasmic part of PGRP-LC.

To gain more mechanistic insight to the interactions between Pirk, Imd, and the cytoplasmic tail of PGRP-LC, we created three Pirk deletion constructs based on the predicted protein structure described in Fig. 5A and tested whether they coimmunoprecipitated with Imd and PGRP-LC. At first, the expression of these deletion mutants was confirmed by Western blotting (data not shown). The N-terminal part of Pirk, Pirk(M1–K51), coimmunoprecipitated neither with Imd nor the PGRP-LC cytoplasmic tail (data not shown). Pirk(D52–V136) encoding the Pirk domain coimmunoprecipitated with Imd (Fig. 7A), whereas the C-terminal part, Pirk(V137–I197), coimmunoprecipitated with cytoplasmic part of PGRP-LC (Fig. 7B). In addition, a weak band was detected when Pirk(D52–V136) coimmunoprecipitated with the cytoplasmic part of PGRP-LC and when Pirk(V137–I197) coimmunoprecipitated with Imd. To test whether these deletion constructs mediated the inhibitory action of Pirk, we monitored Imd pathway activity in S2 cells overexpressing Pirk or Pirk deletion mutants (Pirk(M1–K51), Pirk(D52–V136), and Pirk(V137–I197)) by luciferase assay (Fig. 7C). The N-terminal part of Pirk had no effect on Imd pathway activity. However, both the Pirk domain and the C-terminal part of Pirk abolished Imd pathway activation. These results implicate that Pirk interacts with Imd primarily via the Pirk domain and with the cytoplasmic tail of PGRP-LC via the C-terminal domain. Each of these domains is capable of suppressing the Imd pathway activation.

Pirk-overexpressing flies have lower survival rates upon Gram-negative bacterial infection

To study whether Pirk mediated Imd pathway suppression was sufficient to impair the fly’s immune response in vivo, we monitored the survival of pirk-overexpressing flies upon Gram-negative bacterial infection. Three independent UAS-pirk-V5 fly lines were crossed over C564-GALA driver (induced) or w^{1118} wild-type flies (uninduced), and the week-old healthy offspring were infected with E. coli. C564-GALA crossed over w^{1118} was used as a negative control, and Relish^{209} flies as a positive control. As Fig. 8 shows, the survival rates of the uninduced UAS-pirk-V5 flies and negative control flies did not differ. However, C564-GALA induced flies had significantly lower survival rates compared with the uninduced UAS-pirk-V5 flies. This indicates that overexpression of Pirk suppresses immune response against E. coli. C564-GALA in Drosophila in vivo.

Discussion

A malfunctioning immune system can cause severe damage to the target tissue. NF-kB signaling is of paramount importance in regulating the immune response and thus must be delicately controlled. In mammals, NF-kB activation is regulated by posttranslational modifications of NF-kB and other pathway proteins. The primary NF-kB inhibitors, however, are IκB proteins, which are expressed in a tissue-specific manner and have different affinities to individual NF-kB protein complexes. The Drosophila IκB homologue Cactus (42) inhibits Toll pathway by binding the NF-kB proteins Dif (Dorsal-related immunity factor) and Dorsal. However, the Imd pathway transcription factor Relish is inactive until it is cleaved. Still, signaling events leading to Relish activation must be carefully regulated, which implies that there are other IκB-independent regulatory mechanisms.

In Drosophila, Imd pathway activation is triggered by diaminopimelic acid (DAP)-type peptidoglycan (43), which binds to the receptor PGRP-LC (44). Recent studies implicate that one way of regulating the Imd pathway activity is to dampen the initial stimulus. The extracellular proteins PGRP-LB, PGRP-SC1, and PGRP-SC2 are amidases, which are thought to degrade peptidoglycan and thus act as detoxifying proteins (23, 24). This type of regulation may be especially important when the fly, during all developmental stages, has to adapt to the presence of commensal bacteria in the gut without evoking massive production of antimicrobial peptides or activating apoptosis pathways. Besides the extracellular PGRPs, the membrane-bound PGRP-LF has also been reported to play an immune-suppressing role (45, 25), but the mechanism of this action is still unknown. Another suggested regulatory mechanism of the Imd pathway is proteosomal degradation of either Relish or proteins involved in Relish activation in an SCF (Skp, Cullin, F-box) complex-dependent manner (26). Others have reported that Caspar, the Drosophila FAF1 (Fas associated factor 1) homologue, would suppress Imd pathway activation by preventing Relish cleavage (27). Furthermore, a recent report implies that the active form of Relish, REL-68, can be displaced from the promoter region by a repressor complex formed by JNK and the Jak/STAT pathway transcription factors dA-P-1 and Stat92e together with a high mobility group protein Dsp1 (Dorsal switch protein 1), consequently leading to termination of transcription (28).

In this study we report a novel negative regulatory protein of the Imd pathway that acts on a different level than the previously characterized negative regulators. Pirk is rapidly induced upon the activation of Imd signaling and is likely to mediate its action via direct interaction with Imd and the cytoplasmic part of PGRP-LC. Whereas all the known intracellular components of the Imd pathway are conserved from flies to man, the Pirk domain is not that conserved. We were unable to identify any clear homologues outside the holometabolous insects. There is a putative domain structure (Fig. 5), referred to as the Pirk domain, at the central part of the protein that may be present in other species.
Determining whether any functionally or structurally related proteins exist in mammals may require solving the three-dimensional structure of Pirk. Of note, the receptors of Imd and TNFR signaling pathways are not conserved. Therefore, the interaction of Pirk with PGRP-LC may explain why Pirk is not highly conserved from insects to mammals.

**FIGURE 6.** Pirk is a cytoplasmic protein that coimmunoprecipitates with Imd and the cytoplasmic part of PGRP-LC. A, Pirk is localized in the cytoplasm. S2 cells were transfected with GFP-Pirk fusion protein and GFP-Pirk expression was thereafter analyzed using confocal microscopy. B, Pirk is located upstream of Relish in Imd signaling. Pirk overexpression has no effect on Relish-induced Imd pathway activity. The Imd pathway was activated by overexpressing the active form of Relish (RelΔ29–45), and pirk was expressed under a CuSO₄-responsive promoter. Pathway activity was measured using Ana-luc reporter. C, Pirk negatively regulates JNK pathway. Knocking down pirk increases JNK pathway-mediated expression of puc. S2 cells were treated with pirk dsRNA. JNK signaling was activated with heat-killed E. coli, and puc expression levels were measured by qRT-PCR at the indicated time points. D, Pirk coimmunoprecipitates with Imd and the cytoplasmic tail of PGRP-LC. S2 cells were transfected with myc-tagged constructs of imd, Iap2, Tak1, or the cytoplasmic tail of PGRP-LC (LC(M1–V293)) and V5-tagged pirk. Immunoprecipitation (IP) was done with anti-Myc (α myc) Ab and immunoblotting (IB) with anti-V5 (α V5) Ab. Asterisk at the uppermost panel indicates a nonspecific band.

**FIGURE 7.** Inhibitory action of Pirk is mediated by the Pirk domain and the C-terminal region of Pirk. A and B, Pirk domain coimmunoprecipitates with Imd and C-terminal region of Pirk with cytoplasmic tail of PGRP-LC. In addition, a faint signal is seen when the Pirk domain is immunoprecipitated with PGRP-LC cytoplasmic tail and the C-terminal region of Pirk is immunoprecipitated with Imd. S2 cells were transfected either with V5-tagged Pirk domain encoding Pirk(D52–V136) construct (A) or Pirk C-terminal region encoding Pirk(V137–419) construct (B), and myc-tagged imd or myc-tagged cytoplasmic part of PGRP-LC (LC(M1–V293)). Immunoprecipitation (IP) was performed using anti-Myc (α myc) Ab, and immunoblotting (IB) with anti-V5 (α V5) V5 Ab. C, Pirk domain and the Pirk C-terminal region strongly inhibit Imd signaling. S2 cells were transfected either with an empty vector, full-length pirk, or deletion constructs (Pirk(M1–K51), Pirk(D52–V136), and Pirk(V137–I197)) together with an Ana-luc reporter. Imd pathway was activated by heat-killed E. coli for 24 h.

**FIGURE 8.** Pirk-overexpressing flies are susceptible to E. cloacae infection. Three independent UAS-pirk-V5 lines (UAS-pirk522, UAS-pirk531, and UAS-pirk432) were crossed over C564-GAL4 (induced) or w1118 (uninduced), and the survival of the offspring was monitored after septic injury by E. cloacae. C564-GAL4 over w118 and RelishΔ230 flies were used as negative and positive controls, respectively.
Imd pathway regulation is complex and takes place in many levels. Our current model of Imd pathway regulation is illustrated in Fig. 9. We suggest that Pirk interacts with Imd primarily via the Pirk domain and with the cytoplasmic part of PGRP-LC via the C-terminal region. Curiously, the domain mediating the interaction between PGRP-LC and Imd is not required for signaling (10, 46), which raises a question of whether there still are unidentified components or adaptor proteins essential for signal transduction. Furthermore, the domain of Imd, which mediates interaction with the Pirk domain, is currently not known. Whether a similar regulatory mechanism is applied in mammalian TNFR signaling remains to be elucidated.

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