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Specific Calcineurin Isoforms Are Involved in Drosophila Toll Immune Signaling

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Because excessive or inadequate responses can be detrimental, immune responses to infection require appropriate regulation. Networks of signaling pathways establish versatility of immune responses. Drosophila melanogaster is a powerful model organism for dissecting conserved innate immune responses to infection. For example, the Toll pathway, which promotes activation of NF-κB transcription factors Dorsal/Dorsal-related immune factor (Dif), was first identified in Drosophila. Together with the IMD pathway, acting upstream of NF-κB transcription factor Relish, these pathways constitute a central immune signaling network. Inputs in these pathways contribute to specific and appropriate responses to microbial insults. Relish activity during infection is modulated by Ca\(^{2+}\)-dependent serine/threonine phosphatase calcineurin, an important target of immunosuppressants in transplantation biology. Only one of the three Drosophila calcineurin isoforms, calcineurin A1, acts on Relish during infection. However, it is not known whether there is a role for calcineurin in Dorsal/Dif immune signaling. In this article, we demonstrate involvement of specific calcineurin isoforms, protein phosphatase at 14D (Pp2B-14D)/calcineurin A at 14F (CanA-14F), in Toll-mediated immune signaling. These isoforms do not affect IMD signaling. In cell culture, pharmacological inhibition of calcineurin or RNA interference against homologous calcineurin isoforms Pp2B-14D/CanA-14F, but not against isoform calcineurin A1, decreased Toll-dependent Dorsal/Dif activity. A Pp2B-14D gain-of-function transgene promoted Dorsal nuclear translocation and Dorsal/Dif activity. In vivo, Pp2B-14D/CanA-14F RNA interference attenuated the Dorsal/Dif-dependent response to infection without affecting the Relish-dependent response. Altogether, these data identify a novel input, calcineurin, in Toll immune signaling and demonstrate involvement of specific calcineurin isoforms in Drosophila NF-κB signaling. The Journal of Immunology, 2015, 194: 168–176.

Although higher organisms have acquired adaptive immunity, most metazoans rely on the innate immune system to successfully fight off microbes. Depending on the type of immune insult, immune responses can be local or systemic, and control systems are in check to maintain these. Excessive or inadequate responses can be detrimental to the organism. Insight into the pathways regulating innate immunity is important for identifying how dysregulation causes disease and could provide potential targets for therapeutic intervention.

Drosophila has been instrumental in the genetic dissection of immune defenses postinfection to identify the involved pathways, which turned out to be evolutionarily conserved (1). Two central pathways in Drosophila involved in the response to infection are the TNFR-related IMD pathway and the Toll pathway, known as TLR in mammals. Signaling through IMD or Toll results in the activation of NF-κB transcription factors, which have a key role in mediating inflammatory gene expression. Important transcriptional targets are antimicrobial peptides (AMPs), which help to eliminate infections.

Recognition of pathogen-associated molecular patterns (PAMPs) on microbes, such as peptidoglycan (PGN), occurs through pattern recognition receptors (PRRs). Although TLRs in mammals can serve as a PRR and directly bind to PAMPs, Toll in Drosophila does not. Instead, Toll gets activated upon binding of a cytokine, Spätzle. Spätzle gets cleaved downstream of a protease cascade, which gets activated upon binding of PAMPs (Lys-type PGNs on bacteria or β-glucans on fungi) to specific PRRs. Signaling downstream of Toll promotes activation of NF-κB transcription factor Dorsal and the related transcription factor Dorsal-related immune factor (Dif). In contrast, PRRs that act upstream of IMD mediate responses to diaminopimelic acid–type PGNs, which are primarily found on Gram-negative bacteria. IMD signaling results in activation of NF-κB transcription factor Relish. AMPs downstream of Relish are more effective against Gram-negative bacteria, whereas Dorsal/Dif–specific AMPs help to fight infections with Gram-positive bacteria or fungi (1). In addition to this humoral response to infection, the cellular response by hemocytes (blood cells) plays a central defending role by phagocytosing microbes. The Toll pathway is also involved in this cellular response, because activation of this pathway promotes proliferation of hemocytes (2).

In addition to the canonical IMD and Toll pathways that mediate recognition of microbes to activation of NF-κB transcription factors, there are pathways that modulate their activity. For example, activity of the Ras/MAPK pathway negatively regulates...
IMD-Relish signaling (3). Signaling by the small molecule NO can promote Relish activity independently of IMD (4). Activation of Relish by NO occurs through the calcium-dependent serine/threonine phosphatase calcineurin (5). This phosphatase constitutes a major target for immunosuppression and prevention of graft rejection in mammals. Although the target of calcineurin involved in graft rejection, NFAT, is not present in Drosophila, calcineurin also can regulate the distantly related NF-κB in mammals (6, 7).

Calcineurin acts as a heterodimer, consisting of a catalytic subunit A and a regulatory subunit B. In Drosophila, there are three catalytic subunits: calcineurin A1 (CanA1) and the related and functionally homologous protein phosphatase at 14D (Pp2B-14D) and calcineurin A at 14F (CanA-14F), which are next to each other on the chromosome and probably arose by gene duplication (8). There are two B subunits: CanB and CanB2. CanB is primarily expressed in the brain, and CanB2 is ubiquitously expressed (Flyatlas); therefore, CanB2 probably mediates signaling of the catalytic A subunits in immune tissues. Only CanA1 influences Relish activity in response to infection or to NO signaling, independently of IMD (4, 5), indicating that calcineurin is not part of the canonical IMD-Relish-signaling cascade. Differences in protein sequences may explain why CanA1 is sensitive to NO, whereas Pp2B-14D/CanA-14F are not, but the structural base for this difference is not known. Although treatment with a calcium-mobilizing drug can alter the mobility of Dorsal on SDS-PAGE, possibly via calcineurin (9), a role for calcineurin in Dorsal/Dif-mediated immunity has never been explored (Fig. 1A).

In this study, we examine a potential role for calcineurin in Dorsal/Dif-mediated immunity. We show that, in cell culture, specific isoforms of the calcineurin catalytic subunit, Pp2B-14D and CanA-14F, can mediate nuclear translocation of GFP-tagged Dorsal (GFP-Dif). Coimmunoprecipitation of Pp2B-14D and GFP-Dif suggests that Dorsal may be a direct target for calcineurin. Toll-dependent activation of Dorsal/Dif was attenuated after pharmacologically inhibiting calcineurin or with RNA interference (RNAi) against Pp2B-14D/CanA-14F. In vivo, flies expressing Pp2B-14D/CanA-14F RNAi constructs displayed a decrease in the Toll-dependent immune response and decreased viability postfeeding with Gram-positive bacteria. Expression of active Pp2B-14D was sufficient to induce Dorsal/Dif-dependent expression of LacZ in hemocytes. Together, these data demonstrate the involvement of specific calcineurin isoforms in Dorsal/Dif-mediated immunity. Thus, specific calcineurin isoforms can modulate activity of either Relish or Dorsal/Dif in immunity, providing an additional means of regulation of immunity in Drosophila.

Materials and Methods

Cells, flies, reagents, and Abs

Drosophila S2 cells were cultured in Schneider’s Life Technologies, San Diego, CA) supplemented with 10% heat-inactivated FCS, penicillin, and streptomycin. Cells expressing epidermal growth factor receptor (EGFR)-Toll (10) were a generous gift from the Wasserman laboratory. Transient expression in S2 cells was examined using Cellfectin (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FCS, KCl, and 2 mM CaCl2, supplemented with protease and phosphatase inhibitors. GFP pull-down using GFP-Trap was performed according to the manufacturer’s protocol. Analysis by Western blot and determination of lyses for GFP-Dif mobility were described previously (5).

Luciferase assays

Cells expressing EGFR-Toll were transfected with appropriate plasmids. Six hours after transfection, 20-hydroxyecdysone (1 μM) was added to the cells to sensitize them to immune induction (16). EGF (0.2 ng/ml) was added overnight 30 h after transfection. For pharmacological inhibition of calcineurin, inhibitors were added 30 min prior to adding EGF. Luciferase activity was measured with the Dual Luciferase Assay System Kit (Promega), and samples were normalized with TK-Reporter. Data represent the average of at least three independent experiments.

Infections

Infections of larvae were performed by dipping a needle into a Mucorocus luteus or Erwinia carotovora carotovora 15 (Ecc-15) bacterial solution of 200 OD and pricking the tail of a larva. Larvae expressing GFP under control of the drosomycin promoter (ders-GFP larvae), were used for
analysis of fluorescence. Transgenes were ubiquitously expressed using *daughterless*-Gal4. Larvae were harvested 20 h postinfection and analyzed for GFP fluorescence (in the case of *dsr-GFP* larvae), or RNA was harvested using the RNasy Mini Kit (QIAGEN) for real-time quantitative PCR analysis (RT-qPCR) (15 larvae/pool). RT-qPCR was performed using iQ SYBR Green Supermix and analyzed using MyIQ and iQ5 Real-Time PCR Detection systems (all from Bio-Rad Laboratories). Data represent the ratio of the detected mRNA levels normalized to RP49 mRNA levels as control. For survival experiments of adult flies, 3–4-d-old flies (40 flies/pool) were infected with *M. luteus* by septic injury, maintained at 29°C, and counted every 2 d. Survival experiments were repeated three times.

**Analysis of hemocytes**

Flies expressing active Pp2B-14D, a SERCA RNAi construct, or control flies (w1118) were crossed to either hml-Gal4 or spr-Gal4. For the analysis of β-galactosidase activity, hemocytes of third instar larvae were bled onto chilled coverslips coated with Con A, fixed with 4% formaldehyde, and analyzed for β-galactosidase activity, as described (5).

**Results**

Calcineurin-dependent translocation of GFP-Rel and GFP-Dl

Although calcineurin isoform CanA1 is a downstream target of NO in Relish signaling (5), a possible involvement of NO or calcineurin in Toll-Dorsal/Dif immune signaling has never been explored (Fig. 1A). There are indications for calcineurin involvement in Dorsal signaling: an increase in calcium results in a change in Dorsal phospho-mobility (9) and Dorsal nuclear translocation (17). Changes in mobility of Dorsal were prevented by pretreatment with a serine-threonine phosphatase inhibitor (17). Changes in mobility of Dorsal were prevented by a change in Dorsal phospho-mobility (9) and Dorsal nuclear mobility (2), a possible involvement of NO or thapsigargin treatment (21) (Fig. 1B, left panel). In contrast, a much smaller percentage of cells displayed nuclear GFP-Dl (Fig. 1B, right panel) after NO treatment, whereas thapsigargin-treated GFP-Dl cells showed a percentage similar to that of nuclear GFP-Rel (Fig 1B, right panel). To compare involvement of calcineurin in nuclear translocation of Dorsal and Relish, cells were treated with calcineurin inhibitor FK506 (or cyclosporin A) (Supplemental Fig. 1B) prior to treatment with SNAP or thapsigargin. As shown previously (5), FK506 treatment inhibited GFP-Rel translocation induced by both NO and thapsigargin. In cells expressing GFP-Dl, calcium-induced nuclear translocation also was largely abrogated by FK506. Altogether, these data suggest that the calcineurin isoform(s) activated upon the increase in cytosolic calcium preferentially acts on Dorsal.

**Specific calcineurin isoforms involved in Dorsal translocation**

Next, we tested which isoforms of the catalytic calcineurin subunit, CanA1, Pp2B-14D, and/or CanA-14F, mediate nuclear translocation of Dorsal. The highly homologous Pp2B-14D and CanA-14F are next to each other on the chromosome and probably arose because of gene duplication. They were shown to be functionally redundant (22). Therefore, we studied Pp2B-14D/CanA-14F together. The different isoforms differ at the N or C terminus, and CanA1 differs from Pp2B-14D and CanA-14F throughout the protein. We examined a role for CanA1 or Pp2B-14D/CanA-14F in Dorsal signaling by RNAi using two independent, nonoverlapping dsRNAs to downregulate their expression. Homology between these isoforms and the regions of the dsRNAs targeting the different isoforms are shown in Supplemental Fig. 1A. For Pp2B-14D/CanA-14F, we used dsRNAs that target both isoforms. We verified specific knockdown of calcineurin isoforms using RT-qPCR; RNAi against Pp2B-14D/CanA-14F did not affect mRNA levels of CanA1 and vice versa (Fig. 2A, top panel).

We examined the effect of RNAi against the different calcineurin isoforms on thapsigargin-induced GFP-Dl translocation. Both dsRNAs targeting Pp2B-14D/CanA-14F inhibited GFP-Dl translocation, whereas no effect on thapsigargin-induced translocation was seen with CanA1 RNAi (Fig. 2A, middle panels). With CanA1 RNAi, NO-induced translocation of GFP-Rel was inhibited (data not shown) (5), showing that CanA1 RNAi is sufficient to inhibit CanA1 activity. Quantification of these results is shown below (Fig. 2A, bottom panel).

A serine-rich region in Dorsal is homologous to that in NFAT, and these serines in NFAT are targets of calcineurin. Dephosphorylation of these serine residues promotes nuclear translocation of NFAT, concomitant with a mobility shift of NFAT on Western blot (23). To determine whether Dorsal mobility is affected upon calcineurin activation, as well as which calcineurin isoforms may be involved, we examined changes in GFP-Dl mobility on
FIGURE 2. Distinct calcineurin isoforms are involved in Dorsal/Dif signaling. (A) RNAi-mediated knockdown of calcineurin isoforms (top panel). S2 cells were treated with dsRNA targeting either CanA1 or two different regions of Pp2B-14D/CanA-14F (14D/14F-1, 14D/14F-2) and analyzed for transcript knockdown by RT-PCR. Equal input was verified by RP49 expression. Fluorescent images of S2 cells treated with dsRNA prior to transfection with GFP-Dl and treatment with thapsigargin (1 μM, 1 h) (middle panels). Scale bars represent 10 μM. Percentage of cells displaying nuclear GFP-Dl (bottom left panel). At least 300 cells/point were counted. Data represent the average (± SEM) of at least three independent experiments. Cells expressing GFP-Dl were either left untreated (control) or treated with thapsigargin (thap., 1 μM) in the presence or absence of FK506 (1 nM) and analyzed using Western blot for differences in GFP mobility (upper panel at bottom right). S2 cells were treated with dsRNA targeting either CanA1 or two different regions of Pp2B-14D/CanA-14F (14D/14F-1, 14D/14F-2) for 4 d prior to transfection with GFP-Dl, treatment with thapsigargin (thap., 1 μM, 1 h), and analysis using Western blot (lower panel at bottom right). (B) Fluorescent images of S2 cells transfected with GFP-Dl together with empty vector (control) or active Pp2B-14D (ΔPp2B) (upper panels). Quantification of the percentage of cells expressing nuclear GFP-Dl (lower left panel). At least 300 (Figure legend continues)
SDS-PAGE. Treatment with thapsigargin promoted a downward mobility shift, and this was inhibited by calcineurin inhibitor FK506 (Fig 2A, bottom panel at lower right). When cells were treated with two nonoverlapping dsRNAs against Pp2B-14D/CanA-14F or CanA1, RNAi against Pp2B-14D/CanA-14F, but not against CanA1, was able to interfere with the thapsigargin-induced GFP-DI mobility shift (Fig. 2A, bottom panel at lower right). These data show that calcineurin isoforms Pp2B-14D/CanA-14F mediate thapsigargin-induced GFP-DI translocation, possibly by dephosphorylating Dorsal.

To determine whether a gain-of-function of calcineurin is sufficient to promote nuclear translocation of GFP-DI, we expressed constitutively active ΔΔPp2B-14D (ΔΔPp2B), which lacks the autoinhibitory domain (24). Indeed, expression of ΔΔPp2B-14D resulted in an increase in nuclear GFP-DI (Fig. 2B, upper panels). Quantification of these results is shown in Fig. 2B (lower panel). To determine whether a gain-of-function of calcineurin can alter the mobility of Dorsal on Western blot, we expressed ΔPp2B. Indeed, upon expression of ΔΔPp2B, GFP-DI was shifted downward, possibly suggesting a decrease in the phospho content of Dorsal (Fig. 2B, lower panel).

We next tested whether calcineurin can mediate activity of Dorsal and Dorsal-related transcription factor Dif downstream of Toll. For this purpose, we used S2 cells stably expressing an EGFR–Toll chimeric fusion construct, consisting of the extracellular and transmembrane region of human EGF and the intracellular region of Drosophila Toll. Addition of EGF results in activation of Dorsal/Dif (10). To examine Dorsal/Dif activity, a luciferase construct under the control of a Dorsal/Dif-specific promoter was used (14). Luciferase induced by EGF was largely inhibited upon addition of calcineurin inhibitor FK506 (Fig. 2C), suggesting that Toll activation promotes calcineurin activity involved in Dorsal/Dif signaling. Expression of active Pp2B-14D (ΔΔPp2B), but not of active CanA1 (ΔCanA1), was sufficient to induce luciferase, showing specificity of distinct calcineurin isoforms for inducing Dorsal/Dif activity. The luciferase induction by expression of active Pp2B-14D is lower than by EGF addition, possibly suggesting that additional components of the Toll pathway are required for optimal Dorsal/Dif transcriptional activity. We also tested whether calcineurin RNAi could interfere with Dorsal/Dif activity. We found that two independent dsRNAs targeting Pp2B-14D/CanA-14F attenuated luciferase induction, whereas no effect was found with two independent dsRNAs targeting CanA1. RNAi was not as efficient in EGFR-Toll cells as in regular S2 cells (data not shown), possibly explaining the difference between pharmacological inhibition of calcineurin and RNAi against calcineurin. Activation of the Toll pathway results in phosphorylation and a concomitant increase in phospho-mobility of Dorsal (18). In EGFR-Toll cells, EGF treatment also resulted in an increase in phosphorylation of endogenous Dorsal (Fig. 2C, lower panel). However, upon pharmacological inhibition of calcineurin, this phospho-mobility was no longer seen with EGF. This may indicate that calcineurin activity is required for kinases in the Toll pathway to phosphorylate Dorsal.

To determine whether calcineurin can act on the Toll pathway via direct interaction with Dorsal, we examined whether Dorsal can interact with Pp2B-14D. We also tested interactions of Pp2B-14D with GFP as a negative control and with GFP-VIVIT as a positive control. GFP-VIVIT is GFP fused to a peptide that contains the optimal binding site for calcineurin (13). No interaction was found between GFP and HA-tagged Pp2B-14D, whereas GFP-VIVIT bound HA-Pp2B-14D quite well (Fig. 2D). GFP-DI also coprecipitated Pp2B-14D, with a stoichiometry that appeared comparable to that of GFP-VIVIT and Pp2B-14D. This indicates that calcineurin may bind to and dephosphorylate Dorsal.

Dorsal contains putative consensus calcineurin serines in a serine-rich region (SRR), homologous to that of NFAT, which gets dephosphorylated by calcineurin to subsequently translocate to the nucleus (23), presumably by exposing the nuclear-localization signal (NLS). These serines in the SRR of Dorsal are different from the serines in the Rel homology region (S79, S103, S213, S312, and S317) that get phosphorylated by activation of the Toll pathway (25). In GFP-DI, we mutated three serines in the SRR (S404, S413 and S414) to either alanine (phospho-defective) or aspartic acid (phospho-mimetic), indicated in Fig. 2E (top panel). We examined the effect of these mutations on the mobility of Dorsal, responsiveness to thapsigargin, or Dorsal-dependent luciferase activity. Mutations of the serines to alanine (S3A) resulted in a downward shift of Dorsal on Western blot, whereas the aspartic acid mutant (S3D) displayed an upward shift (Fig. 2E, middle panels). Treatment with thapsigargin resulted in a further downward shift of S3A; however, the downward shift with the S3D mutant was less than that of wild-type (wt) GFP-DI.

We also examined nuclear localization of these mutants: a significant fraction of S3A was present in the nucleus, and this fraction was increased with thapsigargin treatment, but not as much as the control (Fig. 2E, bottom panel, left). In contrast, the S3D mutant was cytoplasmic, and thapsigargin treatment promoted nuclear localization of S3D (Fig. 2E, bottom panel, right). These data indicate that calcineurin activity is required for Dorsal to translocate to the nucleus upon Toll activation.
translocation; however, the total fraction was lower than that of wt Dorsal. This indicates that phosphorylation of these serines are involved in mediating nuclear localization of Dorsal. However, because the response of the S3A and S3D mutants to thapsigargin was attenuated but not inhibited, it indicates that additional serines are involved in mediating the response to calcium. This was shown for NFAT as well (23). When we examined the effect of expressing GFP-DI, as well as its S3A or S3D mutant, on Dorsal-dependent luciferase activity, we found that the increase in activity with GFP-DI was further elevated in the S3A, but not in the S3D, mutant (bottom panel, right). This indicates that serines in the SRR of Dorsal are involved in mediating its transcriptional activity. These findings indicate that the serines in the SRR are target serines for calcineurin and contribute to nuclear localization and activation of Dorsal.

Together, these data suggest that calcineurin can act on the Toll pathway by the binding to and subsequent dephosphorylation of Dorsal, allowing its nuclear translocation.

Specific calcineurin isoforms mediate Dorsal/Dif activity in vivo

Next, we investigated the involvement of calcineurin isoforms Pp2B-14D/CanA-14F in Dorsal/Dif-mediated immune responses in vivo. We used Drosophila larvae because Dorsal mediates immune responses in larvae but not in adults, where Dif alone is required (26). This way, we could extend our S2 cell data, in which we used a Dorsal construct. We tested two independent Pp2B-14D/CanA-14F RNAi foldback constructs (27) that specifically downregulated Pp2B-14D/CanA-14F mRNA without affecting CanA1 mRNA (Fig. 3A, upper panel). We infected larvae with Gram-positive bacteria (M. luteus) to induce the Toll pathway, using septic injury instead of natural infection (oral ingestion), because there was a high variability in oral bacterial intake in the larval population. We examined third instar dros-GFP larvae. Following infection, GFP expression was induced, but this induction was lower when either Pp2B-14D/CanA-14F RNAi construct was expressed (Fig. 3A).

To verify whether expression of endogenous Toll-dependent genes was also dependent on calcineurin, we examined drosomycin, IM1, and IM2 by RT-qPCR. Expression of all of these genes was upregulated postinfection (Fig. 3A, lower panel), and

FIGURE 3. Specific calcineurin isoforms promote activity of Dif/Dorsal in vivo. (A) Third instar control larvae or larvae ubiquitously expressing two independent RNAi constructs targeting Pp2B-14D/CanA-14F (14D/14F RNAi1 or 14D/14F RNAi2) were analyzed for expression of Pp2B-14D/CanA-14F or CanA1, respectively, by RT-PCR (upper left panel). Expression of RP49 was used as a control for equal input. Fluorescent images of uninfected third instar dros-GFP larvae (uninf.), infected third instar dros-GFP larvae (inf.), and dros-GFP larvae ubiquitously expressing Pp2B-14D/CanA-14F RNAi constructs (RNAi1- inf. and RNAi2- inf.) infected with Gram-positive bacteria M. luteus by septic injury (upper right panels). Control larvae or larvae expressing Pp2B-14D/CanA-14F RNAi were infected with M. luteus or Gram-negative bacteria Ecc-15 by septic injury and analyzed for the expression of Dorsal/Dif-dependent genes Drs, IM1, and IM2 or Relish-dependent genes AttA or Dipt by RT-qPCR (lower panel). Expression of RP49 was used as a control. Data are average (± SEM) of at least three independent experiments. (B) Four-day-old male control flies (cg-Gal4/+ or male flies expressing constructs targeting Pp2B-14D/CanA-14F using cg-Gal4, which expresses in the fat body and in hemocytes, were left untreated (control infected and RNAi uninfected) or were infected with M. luteus (control infected and RNAi infected), and their survival was analyzed. Survival curves of control flies with or without infection and RNAi flies without infection were not significantly different from each other, whereas the curve for the infected RNAi flies was (p < 0.001, log-rank test). Data are representative of at least three independent experiments. (C) Induction of Dorsal/Dif-dependent LacZ in hemocytes. Western blot showing expression of active HA-tagged Pp2B-14D (ΔPp2B-HA) third instar larvae, using cg-Gal4 (upper left panel). ΔPp2B-HA expression in larval hemocytes induces expression of Dorsal/Dif-dependent β-galactosidase, D4/Hsp70-LacZ (upper right panel). Hemocytes from D4/Hsp70-LacZ third instar control larvae or D4/Hsp70-LacZ larvae expressing ΔPp2B-HA were analyzed for β-galactosidase activity. Efficacy of SERCA RNAi knockdown (lower left panel). RT-PCR of larvae ubiquitously expressing a SERCA RNAi construct, using daughterless-Gal4. SERCA RNAi induces Dorsal/Dif-dependent β-galactosidase expression (lower right panel). Hemocytes of third D4/Hsp70-LacZ instar larvae with or without SERCA RNAi stained for β-galactosidase activity. Scale bars represent 10 μM. Data are representative of at least three independent experiments. *p < 0.05, Student t test.
this was attenuated by downregulation of $Pp2B-14D/CanA-14F$ expression. We also tested whether $Pp2B-14D/CanA-14F$ RNAi could affect the Relish-dependent response to infection with Gram-negative bacteria Ecc-15. No significant changes were found on levels of the Relish-specific AMPs $diptericin$ and $attacin A$ using RT-qPCR upon downregulation of $Pp2B-14D/CanA-14F$ expression, arguing against a role for these isoforms in Relish signaling.

To examine the consequences of downregulating $Pp2B-14D/CanA-14F$ expression on viability postinfection, we examined survival of these flies after septic injury with $M. luteus$. Whereas no effect on viability was found with infected control flies compared with uninfected flies, $Pp2B-14D/CanA-14F$ RNAi decreased viability postinfection, but not in uninfected flies (Fig. 3B). This demonstrates that $Pp2B-14D/CanA-14F$ are important mediators in the Toll pathway.

To test sufficiency for calcineurin in activating Dorsal/Dif in vivo, we generated flies containing an HA-tagged, active $Pp2B-14D$ transgene ($\Delta Pp2B-HA$) and examined Drosophila hemocytes, in which Toll signaling is known to play a role (2). Expression of this transgene was verified (Fig. 3C, upper panel). To visualize Dorsal/Dif activity, we examined induction of a $\beta$-galactosidase transgene under control of a Dorsal/Dif-specific promoter, $D4/hsp70-LacZ$ (11), containing binding sites specific for Dorsal/Dif (14). Although little $\beta$-galactosidase activity was seen in control hemocytes, a significant fraction of hemocytes expressing active $Pp2B-14D$ displayed $\beta$-galactosidase activity (Fig. 3C, upper panels). Similar observations were made when other fly lines expressing this transgene were used, as well as by using other Gal4 lines with expression in hemocytes (data not shown). This shows sufficiency of calcineurin in inducing Dorsal/Dif activity in vivo.

We also investigated whether increases in cytoplasmic calcium could similarly induce Dorsal/Dif-dependent $\beta$-galactosidase by expressing a SERCA RNAi foldback transgene. Inhibition of SERCA in S2 cells promoted calcineurin-dependent translocation of Dorsal (Fig. 1B), mediated by isoforms $Pp2B-14D/CanA-14F$ (Fig. 2). Efficacy of this $SERCA$ transgene was tested by RT-PCR in larvae ubiquitously expressing the $SERCA$ RNAi construct (Fig. 2B, lower panel). We then expressed the $SERCA$ RNAi transgene in the hemocytes and also observed Dorsal/Dif-dependent induction of LacZ. Similar results were found when a $SERCA$ RNAi construct was used against a different region of $SERCA$ (data not shown). This suggests that elevation of calcium (and subsequent induction of calcineurin activity) can promote Dorsal/Dif activity, although we cannot exclude the calcium-independent effects of $SERCA$ knockdown, such as endoplasmic reticulum stress.

Thus, specific calcineurin isoforms are an important input for modulating the activity of either Relish or Dorsal/Dif without cross-activation between these NF-κB pathways. Involvement of calcineurin in Drosophila NF-κB immune signaling is summarized in a model (Fig. 4). Specific inputs of calcineurin help to achieve appropriate and specific immune activation.

**Discussion**

We show that activity of the two distinct pathways of the NF-κB family in Drosophila, IMD-Relish and Toll-Dorsal/Dif, can be modulated by a calcium-dependent serine/threonine phosphatase, calcineurin. However, specificity of calcineurin activity in these pathways is achieved by distinct isoforms of the catalytic calcineurin subunit A interacting with each pathway. Calcineurin isoform CanA1 modulates activity of Relish, whereas the functionally homologous and related $Pp2B-14D$ and $CanA-14F$ mediate activity of Dorsal/Dif.

Previous work showed that activity of CanA1 modulates activity of Relish during infection (5) and that CanA1 acts downstream of NO. When we set out to analyze whether NO also could be involved in Dorsal/Dif signaling, we found that nuclear translocation of GFP-Dl by NO was much smaller compared with that of GFP-Rel. However, treatment with a drug that promotes an elevation in intracellular calcium and subsequent activation of calcineurin resulted in much higher levels of GFP-Dl nuclear translocation. This suggests that calcineurin can mediate Dorsal signaling but via a different isoform than CanA1. Subsequent RNAi experiments showed that the calcineurin isoforms responsible for GFP-Dl translocation were the functionally homologous and related $Pp2B-14D$ and $CanA-14F$. We show in this study that RNAi targeting $Pp2B-14D/CanA-14F$ downregulates the Dorsal/Dif-mediated response to Gram-positive bacteria, whereas the Relish-dependent response to Gram-negative bacteria was unaffected (Fig. 3A). This decrease in the response by $Pp2B-14D/CanA-14F$ RNAi was accompanied by a decrease in survival (Fig. 3B). This indicates that, in vivo, $Pp2B-14D/CanA-14F$ are important in mediating Toll-dependent signaling and do not interact with IMD signaling.

To our knowledge, this is the first report implying calcineurin in Toll-mediated immune signaling and demonstrating involvement of specific calcineurin isoforms in this pathway. Thus, calcineurin acts in both IMD and Toll signaling, and specificity of calcineurin in these pathways is acquired by specific isoforms acting on Dorsal/Dif or Relish. Only CanA1 can act downstream of NO, and it modulates Relish signaling in response to infection or to NO (5). NO is generated during infection with Gram-negative bacteria (4), $Pp2B-14D$ and $CanA-14F$ are activated in response to an increase in calcium, and they can promote nuclear translocation.

**FIGURE 4.** Model for involvement of specific calcineurin isoforms in Drosophila NF-κB signaling. Specific isoforms of the catalytic calcineurin subunit $A$ mediate activity of either Relish or Dorsal/Dif. In the IMD pathway, a specific calcineurin isoform, CanA1, gets activated upon generation of NO during infection and promotes nuclear localization of Relish, independently of IMD. In the absence of CanA1, Relish-dependent responses to infection are attenuated. In contrast, activation of Dorsal/Dif downstream of Toll is dependent on two homologous calcineurin isoforms: $Pp2B-14D$ and $CanA-14F$. Activity of these calcineurin isoforms induces nuclear localization and contributes to activation of Dorsal. In the absence of $Pp2B-14D/CanA-14F$, responses to infection with Gram-positive bacteria are attenuated, and viability is decreased, demonstrating the importance of these isoforms in Dorsal/Dif activity. Because $Pp2B-14D/CanA-14F$ are dependent on an increase in cytoplasmic calcium for their activation, we infer that activation of the Toll pathway is accompanied by calcium mobilization, possibly via the CRAC calcium channel (Supplemental Fig. 1B).
and activation of Dorsal in cell culture downstream of Toll activation (Fig. 2A–C). In vivo, RNAi against Pp2B-14DCanA-14F is sufficient to dampen the Dorsal/Dif-dependent response to infection with Gram-positive bacteria, concomitant with a decrease in survival. These findings suggest that activation of Toll results in mobilization of calcium and subsequent activation of Pp2B-14DCanA-14F.

Does calcineurin contribute to Toll signaling by directly acting on Dorsal? Pp2B-14D does coprecipitate with Dorsal. There is a putative calcineurin docking site (PxIxIT) (13) in Dorsal (aa 47–52; PYVKIT). However, mutation of this site did not abrogate the association between Dorsal and Pp2B-14D (data not shown). Recent analysis of docking sites on calcineurin substrate demonstrated that the calcineurin docking site is a bit more versatile (28), so additional sites in Dorsal (such as aa 15–20; PAVDGQ) may serve as a calcineurin docking site. Although we do not know whether the interaction between Dorsal and calcineurin is direct, it may allow calcineurin to dephosphorylate serine residues on Dorsal, which is the main residue of Dorsal that gets phosphorylated (25). To examine whether Dorsal is a target for calcineurin, we mutated target phosphorylation sites for calcineurin on Dorsal in an SRR that is adjacent to the NLS (Fig. 2E), homologous to those on NFAT. Action of calcineurin on NFAT results in unmasking of an NLS, resulting in nuclear translocation of NFAT (23). Similarly, Relish also contains calcineurin target sites surrounding the NLS. Mutation of serines to alanine of putative calcineurin target sites on Relish yielded a constitutively nuclear protein (5). Mutation of serines in the SRR of Dorsal demonstrated involvement of these serines in mediating nuclear localization of Dorsal and contribution to its activity (Fig. 2E). Previous work in Drosophila showed that Toll-dependent phosphorylation occurs in the N-terminal Rel homology domain and that six serines in this domain are phosphorylated (25). However, these are not calcineurin consensus sites. Our data and work on NFAT (29) suggest that calcineurin acts on residues in a SRR. We also show that pharmacological inhibition of calcineurin inhibits Toll-dependent phosphorylation of Dorsal and Dorsal/Dif activity (Fig. 2C). What are the events that contribute to the phosphorylation state of Dorsal after Toll activation? A model explaining our observations is that, upon Toll activation, calcineurin gets activated and dephosphorylates Dorsal on serine residues (in the SRR). Calcineurin activity appears to be required for Toll-dependent kinases to phosphorylate Dorsal on different serines in the Rel homology domain to fully activate Dorsal. Although we only examined the mobility of Dorsal, and not Dif, in response to calcineurin activity, Dif is probably also subject to regulation by calcineurin, because blocking calcineurin in cells attenuated Dis/Dorsal-dependent transcription.

It is not known why NO only signals through CanA1 and not through Pp2B-14DCanA-14F. Although the catalytic domains of these subunits are highly conserved (Supplemental Fig. 1A), the N and C termini of CanA1 are rich in serine and threonine, possibly allowing for posttranslational modification downstream of NO. Differences in calcineurin sequence also may account for the differences in substrate specificity. Although Pp2B-14D and CanA-14F are functionally redundant (22), there may be differences in their substrate specificity, because they differ in their N and C termini (Supplemental Fig. 1A). In mammals, the catalytic A subunits of calcineurin are also most variable in their N and C termini. A proline-rich region in the N terminus of the catalytic calcineurin subunit CanA β is involved in the recognition of NFAT (30). The different isoforms appear to have different distinct targets in vivo, with CanA β being the main regulator of NFAT activity (reviewed in Ref. 31). Isoform-specific inhibitors might be able to circumvent the significant side effects occurring with the current immunosuppressing drugs that target all calcineurin isoforms.

The calcium channel that mediates influx of calcium that promotes activation of NFAT is called Ca2+ release-activated Ca2+ (CRAC) channel. Patients with mutations in this channel are severely immunocompromised. Interestingly, the identity of this channel, Orai1, was first identified in a Drosophila RNAi screen in which S2 cells expressing human NFAT were examined for mediators of nuclear translocation (32), suggesting evolutionary conservation of calcium and calcineurin signaling. Dysregulation of calcium homeostasis, resulting in increased intracellular calcium and subsequent calcineurin activation, may result in aberrant Dorsal/Dif activation. This is supported by data showing that ΔPp2B-expressing larvae, as well as SERCA RNAi-expressing larvae, had increased levels of β-galactosidase under control of a Dorsal/Dif-dependent promoter (Fig. 3B). Moreover, the number of hemocytes was increased in these larvae (data not shown). An increase in hemocyte numbers also may be mediated by Dorsal/Dif, because constitutive activation of the Toll pathway results in overproliferation of hemocytes (2). Preliminary research suggests that the channel involved in calcium increase resulting in nuclear translocation of Dorsal, as well as Dorsal/Dif-dependent transcription, may indeed be Orai1. Pharmacological inhibition of CRAC, as well as RNAi against Orai1, inhibited Dorsal/Dif-dependent translocation in response to thapsigargin (Supplemental Fig. 1B, left panel). Moreover, pharmacological inhibition of this channel or Orai RNAi attenuated Dorsal/Dif-dependent induction of luciferase downstream of Toll (Supplemental Fig. 1B, right panel), suggesting putative involvement of this channel in Dif/Dorsal-dependent immunity.

Altogether, calcineurin is an important phosphatase involved in specific immune pathways in both flies and mammals. Specifically targeting the isoforms involved in these particular pathways, as well as targeting phosphorylation sites of calcineurin substrates in these pathways, may be a way to modulate immune activation.

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Disclosures
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