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The *Drosophila* Atypical Protein Kinase C-Ref(2)P Complex Constitutes a Conserved Module for Signaling in the Toll Pathway

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Recent results showed the critical role of the mammalian p62-atypical protein kinase C (aPKC) complex in the activation of NF-κB in response to different stimuli. Here we demonstrate using the RNA interference technique on Schneider cells that the *Drosophila* aPKC (DaPKC) is required for the stimulation of the Toll-signaling pathway, which activates the NF-κB homologues Dif and Dorsal. However, DaPKC does not appear to be important for the other *Drosophila* NF-κB signaling cascade, which activates the NF-κB homologue Relish in response to lipopolysaccharides. Interestingly, DaPKC functions downstream of the nuclear translocation of Dorsal or Dif, controlling the transcriptional activity of the Drosomycin promoter. We also show that the *Drosophila* Ref(2)P protein is the homologue of mammalian p62 as it binds to DaPKC, its overexpression is sufficient to activate the Drosomycin but not the Attacin promoter, and its depletion severely impairs Toll signaling. Collectively, these results demonstrate the conservation of the p62-aPKC complex for the control of innate immunity signal transduction in *Drosophila melanogaster*.

The atypical protein kinase C (aPKC) subfamily is composed of two members: ζPKC and λ/ιPKC. These isoforms are characterized by their insensitivity to classical PKC activators, such as diacylglycerol and Ca^{2+}, in contrast to the more typical PKCs, which contain motifs in their regulatory domains that make them targets for those second messengers (25). Both aPKCs have been shown to be involved in several cellular functions, including growth and survival, as well as in the establishment and maintenance of epithelial cell polarity (24). These kinases have also been implicated in the regulation of NF-κB, where they are thought to be essential in the regulation of the phosphorylation of the RelA subunit of this transcription factor (20, 24). The mechanism by which the aPKCs can regulate these different signaling pathways is not completely clear. However, the existence of scaffold proteins may explain how a single PKC subtype can be involved in different signaling cascades. Thus, through their interaction with p62, the aPKCs are located in the NF-κB pathway (28–30), while their binding to MEK5 (7) may serve to place the aPKCs in the BMK1/ERK5 mitogenic signaling cascade that likely regulates c-Jun expression. In addition, the interaction with Par-6/ASIP involves the aPKCs in the control of cell polarity. p62, Par-6, and MEK5 harbor in their respective amino-terminal regions a small sequence of acidic amino acids, termed AID (for aPKC interaction domain), that is required for the interaction of these adapters with the aPKCs (24). The AID region is a subtype of the OPCA motif, which groups AID-related sequences, such as the octicosapeptide repeat and the Phox and CDC motifs (PC) (26). The *Drosophila* homologue of Par-6 has been identified, which indicates that the Par-6–aPKC cassette is also conserved in flies (16, 22, 27, 37, 40). Interestingly, the *Drosophila* orthologue of p62 has not been investigated yet.

The role of the aPKCs in the NF-κB pathway has been established by using different and independent strategies, such as the microinjection of inhibitor peptides, the use of antisense oligonucleotides, and the transfection of dominant negative mutants (24). Recent results from this laboratory demonstrate that embryonic fibroblasts from $\zeta PKC^{-/-}$ mice show impairment in the NF-κB pathway, which affects the ability of this factor to activate transcription (20). This finding demonstrates an essential and nonredundant role of ζPKC in this important cascade, which cannot be compensated for by the presence of the other aPKC isoform, λ/ιPKC.

Drosophila melanogaster encodes only one aPKC (DaPKC), making it a simpler system for investigation of the role of aPKC in different signaling pathways. Also, studies using *Drosophila* cell cultures are particularly powerful because the use of the RNA interference technique (RNAi) has proven to be an efficient way to selectively deplete cells of signal transduction proteins. In particular, *Drosophila* cell culture is an excellent system to investigate the immune signaling pathways which activate the *Drosophila* NF-κB homologues, as there is a remarkable degree of homology with mammalian systems (33). For example, Dorsal and Dif, the *Drosophila* homologues of RelA, have been shown to play a critical role in the control of innate immunity and Dorsal also plays a role in early embryonic patterning (14, 33). Both Dorsal and Dif are retained in the cytosol by the IκB homologue Cactus, whose phosphorylation and subsequent degradation release both transcription factors from the cytosolic complex, allowing them to translocate to the nucleus (10, 33). The identity of the kinase responsible for the phosphorylation of Cactus is still unclear, but it

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has been well established that this event depends on the kinase Pelle, which is similar to interleukin-1 receptor-associated kinase (IRAK), a critical component of the interleukin-1–NF-κB signaling pathway (14, 33). Through the adapter Tube and the *Drosophila* homologue of MyD88 (12, 38), Pelle links Cactus degradation to the Toll receptor signaling pathway in *Drosophila* (33). In mammals, IRAK binds TRAF6, which in turn is responsible for NF-κB and Jun kinase (JNK) activation (4, 23). The *Drosophila* homologue of TRAF6 may be DTRAF2, which has recently been implicated in the activation of the Dorsal pathway (32).

Parallel to Toll signaling another cascade is critical for the insect immune response. This pathway requires a different Rel family member, Relish, which is the fly homologue of p100/p105 (9, 18, 34, 36). The mechanism of activation of this Rel protein is different from that of classical RelA/p50 in mammals and those of Dorsal and Dif in *Drosophila*. Relish is composed of an N-terminal Rel homology domain followed by a C-terminal IκB-like sequence; its endoproteolytic cleavage is triggered by an IκB kinase (IKK) enzymatic activity similar to that of the mammalian signalsome complex responsible for the phosphorylation of IκB and p100 (34). This Relish pathway is activated by infection by gram-negative bacteria or lipopolysaccharide (LPS) treatment, while the Toll pathway is more critical for immunity to fungi and gram-positivie bacteria (33).

In this study, we sought to determine whether DaPKC plays a role in either of the two *Drosophila* NF-κB cascades, as well as whether the p62-aPKC cassette is conserved in this organism.

MATERIALS AND METHODS

Reagents and cell culture. S2 cells were purchased from Invitrogen. S2tpll cells are stable clones engineered to assay the Toll pathway by Cu^{2+} addition (34). Cells were grown at 25°C in Schneider medium (Gibco BRL) supplemented with 10% inactivated fetal calf serum. The monoclonal 12CA5 anti-hemagglutinin (HA) and anti-Flag antibodies were from Boehringer and Sigma, respectively. The rabbit anti-aPKC (C-20) and anti-Myc were from Santa Cruz Biotechnologies. The Dorsal and the Cactus monoclonal antibodies were a gift from Ruth Steward. The Relish antibody was the anti-C described previously (9, 36).

Plasmids. The templates for the RNAi experiments were constructed by cloning the first 600 bp of *Drosophila* aPKC and the first 300 bp of the Ref(2)P coding regions in pGEMT vectors (Promega). The luciferase reporter constructs with the Drosomycin and Attacin promoters were a generous gift from J. M Imler and were described previously (38). For expression in insect cells, the Myc-Ref(2)P plasmid was constructed by isolating S2 cDNA by reverse transcription and subcloning it into the inducible plasmid pMT/V5 (Invitrogen); the *Drosophila* aPKC cDNA was subcloned into the same vector. The human ιPKC cDNA was subcloned into the pPAC vector. HA-DTRAF2 was a generous gift from J. L. Manley (32). For expression in mammalian 293 cells, Myc-Ref(2)P, HA-Ref(2)P, and Flag-TRAF2 were constructed by subcloning the coding sequences into the pCDNA3 vector (Invitrogen).

RNAi. The RNAs of aPKC and Ref(2)P were synthesized with the SP6 and T7 RiboMAX RNA production system (Promega). The RNA single strands were hybridized by heating them for 30 min at 65°C and then cooling them slowly to room temperature. S2 or S2tpll cells were plated 1 day before transfection by the calcium phosphate precipitation technique, with 15 μg of double-stranded RNA (dsRNA) in a mix with a total of 50 μg of DNA and RNA per each 9×10^6 cells. The next day, cells were washed four times with phosphate-buffered saline (PBS) and ecdysone was added to a final concentration of 1 μM.

Luciferase reporter assays. Cells were seeded in six-well plates 1 day before transfection, and then they were transfected with 0.5 μg of reporter plasmid and 2 μg of dsRNA. For the DTRAF2 and Ref(2)P expression experiments, an additional 1 to 3 μg of expression plasmids was added. The total amount of RNA and DNA per well was adjusted to 8 μg, and a *Renilla* reporter was used as a control. All transfections were done in triplicate. Cells were induced with copper sulfate to a final concentration of 500 μM for either 12 h to express HA-Ref(2)P or 1 to 5 h to induce the Toll pathway. Then cells were harvested and washed twice with PBS, and the luciferase and *Renilla* activities were determined by the Dual-Luciferase reporter assay from Promega.

Coimmunoprecipitations and Western blot analyses. For immunoprecipitations, transfected 293 or S2 cells were lysed in PD buffer (40 mM Tris-HCl [pH 8], 500 mM NaCl, 0.1% NP-40, 6 mM EDTA, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM phenylphosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 M phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml, 1 μg of leupeptin/ml, 1 μg of pepstatin/ml, 1 mM dithiothreitol) and incubated with protein A or protein G beads for 2 h. Clarified lysates were incubated overnight with the appropriate antibody (anti-HA antibody, a monoclonal anti-Flag antibody, or a polyclonal anti-ζPKC antibody). The immune complexes were recovered by the addition of protein A or G beads. After extensive washing, beads were boiled and resolved on 8% polyacrylamide gels. Proteins were transferred onto poly(vinylidene fluoride) membranes by electroblotting and then probed with the corresponding antibody. The membrane localization of ζPKC in Ref(2)P RNAi-treated cells was determined as described previously (28).

Cytosolic and nuclear fractionation. About 9×10^6 transfected cells were harvested, washed twice with PBS, and incubated for 5 min at 4°C in 1 ml of buffer A (10 mM HEPES [pH 8], 50 mM NaCl, 0.5 M sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5% Triton X-100, 7 mM β-mercaptoethanol, and protease inhibitors). The lysate was spun, and the supernatant constituted the cytosolic fraction. The pellet was washed with buffer B (10 mM HEPES [pH 8], 50 mM NaCl, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 25% glycerol, 7 mM β-mercaptoethanol, and protease inhibitors), incubated for 30 min at 4°C in 30 to 80 μl of buffer C (10 mM HEPES [pH 8], 350 mM NaCl, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 25% glycerol, 7 mM β-mercaptoethanol, and protease inhibitors), and centrifuged for 15 min at maximum speed to obtain the nuclear fraction.

RT-PCRs. The total RNA was extracted from transfected S2 or S2tpll cells by using ULTRASPEC reagent according to the instructions of the manufacturer (Biotecx). The Drosomycin, Diptericin, Ref(2)P, and Dactin mRNA levels were determined by a single-step reverse transcription-PCR (RT-PCR) with the following oligonucleotides: for Drosomycin, 5′-CATTTACCAAGCTCCGTGAG-3′ and 5′-GTAGTGGAGAGCTAAACGCG-3′; for Diptericin, 5′-GGCTTCAATTGAGAACAACTG-3′ and 5′-CTAGACTCGGATACCAATCG-3′; for Ref(2)P, 5′-CCACAAGCTGAGCCCACTGTTACC-3′ and 5′-TTGAATATGAATATTTAG TTGCGG-3′; and for Dactin, 5′-CGCTGAACCCCAAGGCCAAC-3′ and 5′-TCATGATGGAGTTGTAGGTGGTCTC-3′. Thirty nanograms of total RNA was used as a template, and conditions were done in the linear range.

In vitro phosphorylation of Dif. Three micrograms of recombinant glutathione *S*-transferase (GST)-Dif was incubated at 30°C for 30 min in 20 μl of assay buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM dithiothreitol, and 50 μM ATP in the presence of recombinant baculovirus-expressed ζPKC (30 ng).

RESULTS

The down-regulation of DaPKC selectively impairs the activation of the Toll pathway in *Drosophila* Schneider cells. In order to address the potential role of DaPKC in the *Drosophila* innate immunity system, we depleted DaPKC levels using RNAi in Schneider S2 cells that had been engineered to assay the Toll-signaling pathway (34). These cells had been stably transfected with a Torso-Pelle fusion construct controlled by the metallothionein promoter (S2tpll). This construct creates a constitutively active Pelle kinase that activates the Cactus/Dorsal and Cactus/Dif cascades upon its expression following the addition of Cu^{2+} to S2tpll cell cultures. To stimulate the Relish pathway, cells were treated with LPS. The activation of the Toll pathway was monitored by quantifying the induction of the antimicrobial peptide gene Drosomycin. The activation of the Relish cascade was determined by monitoring the induction of the antimicrobial peptide gene Diptericin. S2tpll cells were transfected with DaPKC dsRNA, which leads to a substantial reduction of the DaPKC protein levels (Fig. 1A). These cells were either stimulated with LPS or Cu^{2+} for 3 h or left un-

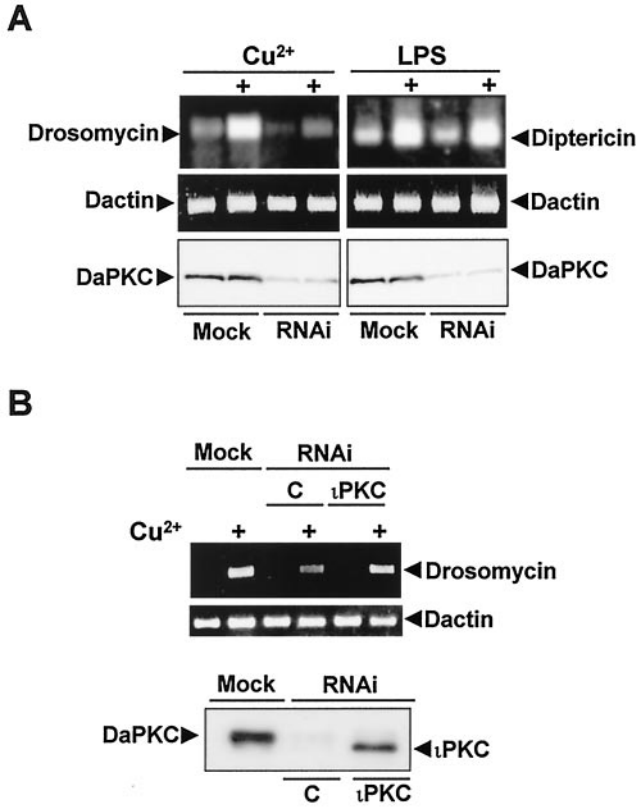

FIG. 1. DaPKC is essential for Drosomycin transcription. (A) S2tpll cells were transfected with DaPKC dsRNA (RNAi) or left untreated (Mock), after which they were stimulated with either Cu^{2+} (left panel) or LPS (right panel) and the transcriptional activation of Drosomycin (left panel) or Diptericin (right panel) was determined by RT-PCR. Dactin mRNA levels were also determined as a control for RNA loading. The levels of DaPKC were determined in parallel cell extracts by immunoblotting. The results shown are representative of results from at least two other experiments. (B) In another experiment, S2tpll cells that were transfected with DaPKC dsRNA were transfected with either the plasmid control, C, or an expression vector for human ιPKC, after which they were stimulated with Cu^{2+} and the transcriptional activation of Drosomycin was determined by RT-PCR. Dactin mRNA levels were also determined as a control for RNA loading. The lower panel, which shows the results of immunoblotting with an antibody that cross-reacts with both aPKCs, demonstrates the effective depletion of DaPKC and the expression of human ιPKC. Note that ιPKC runs faster than DaPKC in this gel.

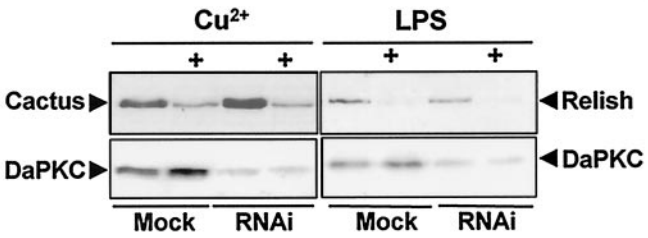

FIG. 2. DaPKC is not required for Cactus or Relish degradation. S2tpll cells were transfected with DaPKC dsRNA (RNAi) or not transfected (Mock) and stimulated as described above, after which the levels of Cactus (left panel), Relish (right panel), and DaPKC (both panels) were determined by immunoblotting with the corresponding antibodies. The results shown are representative of results from at least three other experiments.

stimulated, and the transcriptional activation of Diptericin or Drosomycin, respectively, was determined by semiquantitative RT-PCR. Results shown in Fig. 1A demonstrate that the reduction of DaPKC levels leads to a severe inhibition of the torso-pelle-induced transcription of Drosomycin, with no effect on the LPS-mediated induction of Diptericin. The activation of *Drosophila* Jun kinase by the addition of Cu^{2+} to the S2tpll cells is not affected by the depletion of DaPKC levels (data not shown). These observations strongly suggest that DaPKC plays an essential and selective role in the Toll pathway. When the human aPKC ιPKC was transfected into cells treated with DaPKC dsRNA, the induction of Drosomycin was largely restored (Fig. 1B).

Depletion of DaPKC does not affect Cactus or Relish degradation. The activation of the Toll pathway induces the degradation of Cactus. To determine whether DaPKC acts upstream or downstream of this step in the pathway, S2tpll cells transfected or not transfected with DaPKC dsRNA were induced to express the Torso-Pelle construct or were stimulated with LPS for 3 h and the degradation of Cactus and the cleavage of Relish were determined by immunoblot analysis of cell extracts. The expression of Torso-Pelle (Fig. 2), but not the addition of LPS (data not shown), provokes a reproducible degradation of Cactus which is not affected by the reduction in DaPKC levels (Fig. 2). Conversely, LPS addition (Fig. 2) but not Torso-Pelle expression (data not shown) promotes the cleavage of Relish, which is not affected by the reduction of DaPKC levels (Fig. 2). Collectively, these results indicate that DaPKC plays an essential role in the Toll-Dorsal/Dif immune pathway, acting downstream of Cactus degradation.

Dorsal and Dif nuclear translocation in DaPKC-depleted cells. Upon the degradation of Cactus, Dif and Dorsal are released and translocate to the nucleus (33). To determine if the inhibition of Drosomycin transcription detected in the DaPKC-depleted cells could be accounted for by a decrease in Dorsal nuclear translocation, cells were treated as described above, after which they were fractionated and Dorsal levels in the nuclear fraction were determined. Results shown in Fig. 3A demonstrate that the lack of DaPKC has little effect on the accumulation of nuclear Dorsal in response to torso-pelle expression. To address whether the nuclear translocation of Dif could be affected by the depletion of DaPKC levels, S2tpll cells were transfected with a Flag-tagged Dif expression vector along with a plasmid for Cactus, whose expression impedes the unregulated nuclear translocation of Dif, with or without DaPKC dsRNA, after which cells were induced to express the Torso-Pelle construct and the nuclear translocation of Flag-Dif was determined. Interestingly, the depletion of DaPKC levels has little effect on the induced nuclear translocation of Dif (Fig. 3B).

The down-regulation of DaPKC inhibits Drosomycin promoter transcriptional activity. The results obtained so far strongly suggest that DaPKC is required for the activation of Drosomycin transcription at a level that is downstream of Cactus and the translocation of Dorsal or Dif. In order to demonstrate that this is actually the case, S2tpII cells were transfected with a Drosomycin promoter luciferase reporter

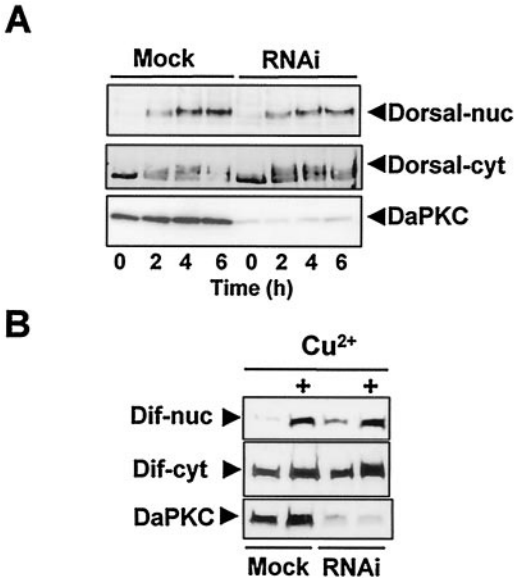

FIG. 3. DaPKC is not required for the nuclear translocation of Dorsal or Dif. S2tpll cells were transfected (B) or not transfected (A) with a Flag-tagged Dif expression vector along with a Cactus plasmid with (RNAi) or without (Mock) DaPKC dsRNA, after which they were stimulated or not stimulated with Cu^{2+}, and the levels of nuclear Dorsal (Dorsal-nuc) (A), cytosolic Dorsal (Dorsal-cyt) (A), nuclear Flag-Dif (Dif-nuc) (B), cytosolic Flag-Dif (Dif-cyt) (B), and total DaPKC (A and B) were determined by immunoblotting. The results shown are representative of results from at least three other experiments.

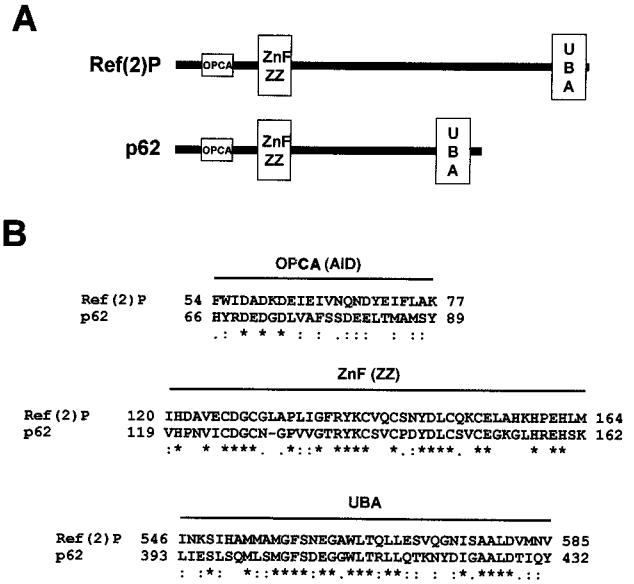

FIG. 5. Sequence and structure conservation between Ref(2)P and p62. (A) Schematic representation of Ref(2)P and p62 sequences. OPCA is the sequence with homology to the AID domain, ZnF (ZZ) is the atypical ZZ zinc finger domain, and UBA is the ubiquitin-associated domain. (B) Sequence alignments of the OPCA, ZnF, and UBA domains of Ref(2)P and p62 were done with the Clustal program.

construct either with or without DaPKC dsRNA, after which cells were induced or not induced with Cu^{2+} and the luciferase activities were determined for all samples. Consistent with the RT-PCR results, the activation of the Drosomycin promoter by Torso-Pelle was severely inhibited by the down-regulation of DaPKC (Fig. 4). As a control, cells were transfected in parallel with a luciferase reporter construct under the control of the Attacin promoter that is selectively activated by LPS (38). In marked contrast to what occurred with the Drosomycin promoter, the down-regulation of DaPKC did not inhibit the transcriptional activity of the Attacin promoter activated by LPS (Fig. 4). This finding is in good agreement with the notion that DaPKC plays a selective and critical role in the Toll pathway in *Drosophila* cells.

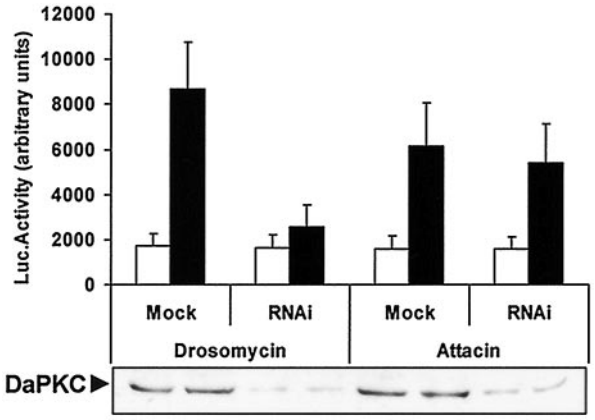

FIG. 4. DaPKC is required for the activation of the Drosomycin promoter. S2tpll cells were (RNAi) or were not (Mock) transfected with DaPKC dsRNA along with either Drosomycin or Attacin luciferase reporter vectors, after which cells were stimulated (black bars) with Cu^{2+} (for the Drosomycin promoter) or LPS (for the Attacin promoter) or were left unstimulated (white bars), and the luciferase activities of cell extracts were determined. The results shown are representative of results from at least three other experiments. A representative immunoblot is shown for endogenous DaPKC.

Ref(2)P is critical for the activation of the Drosomycin promoter. Recent studies from this laboratory have implicated the aPKC adapter p62 in NF-κB activation in mammalian cells (29). p62 interacts physically and functionally with TRAF6, which is required for NF-κB activation in interleukin-1-stimulated cells (23). A search of the Flybase data bank reveals the existence of a potential orthologue of p62, named Ref(2)P, that has an overall structure similar to that of p62, including the AID sequence, a putative ZZ zinc finger, and a C-terminal ubiquitin-associated domain (Fig. 5). The overall similarity at the amino acid level between Ref(2)P and human p62 is 23.9%, and the identity is 17.9%. This is comparable to the overall homology between human TRAF6 and DTRAF2 (31.1% similarity and 21.9% identity) or between human MyD88 and DMyD88 (22.1% similarity and 14.3% identity). The exact function of Ref(2)P as well as its mechanism of action is still unclear, although earlier studies indicated that it may play a role in the normal replication of some strains of sigma virus (5, 6, 39, 42). Based on the role of p62 in mammalian cells and its homology with Ref(2)P, we sought to determine whether the overexpression of Ref(2)P was sufficient to activate the Drosomycin promoter. Thus, S2 cells were

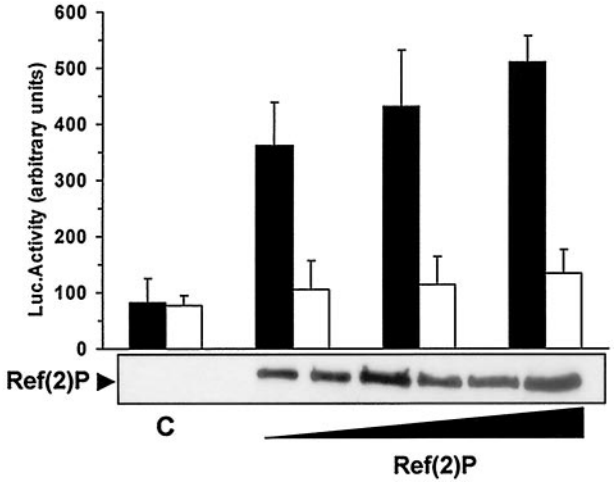

FIG. 6. Ref(2)P expression activates the Drosomycin promoter. S2 cells were transfected with the Drosomycin (black bars) or the Attacin (white bars) luciferase reporter as described above, along with increasing amounts (1, 3, and 10 μg) of a Ref(2)P expression vector, and the luciferase (Luc.) activities of cell extracts were determined. Results are the means ± standard deviations (SD) of results from three independent experiments with duplicate incubations. A representative immunoblot for Ref(2)P that was subjected to the corresponding anti-tag antibody is shown below the graph.

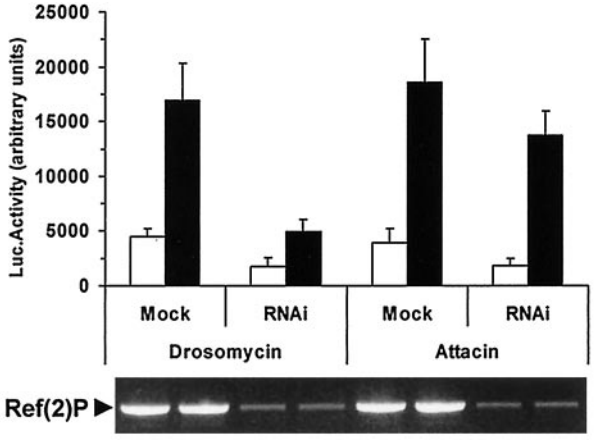

FIG. 7. Ref(2)P is required for the activation of the Drosomycin promoter. S2tpll cells were (RNAi) or were not (Mock) transfected with Ref(2)P dsRNA along with either Drosomycin or Attacin luciferase reporter vectors, after which cells were stimulated (black bars) with Cu^{2+} (for the Drosomycin promoter) or LPS (for the Attacin promoter) or were left unstimulated (white bars), and the luciferase (Luc.) activities of cell extracts were determined. Results are the means ± SD of results from three independent experiments with duplicate incubations. A representative RT-PCR gel for Ref(2)P is shown below the graph.

transfected with either the Drosomycin or the Attacin reporter constructs as described above, along with a plasmid control or increasing amounts of a Ref(2)P expression vector, and the luciferase activities were determined. Interestingly, the expression of Ref(2)P is sufficient to activate the Drosomycin but not the Attacin promoter (Fig. 6).

The antisense-mediated depletion of p62 in mammalian cells leads to an inhibition of NF-κB activation in response to different stimuli (29, 41). In order to determine whether Ref(2)P is selectively required for the activation of the Drosomycin promoter, S2tpll cells were transfected with the Drosomycin or the Attacin reporter and treated, or not treated, with Ref(2)P dsRNA, and then they were either left untreated or induced with Cu^{2+} or LPS and the luciferase activity was measured. Consistent with the notion that Ref(2)P may be the *Drosophila* p62 homologue, the depletion of Ref(2)P leads to a severe reduction of the activation of the Drosomycin but not the Attacin promoter (Fig. 7). In addition, when Drosomycin and Diptericin mRNA levels were determined in Ref(2)P-depleted cells, it was clear that the induction of Drosomycin transcription by Torso-Pelle was significantly reduced whereas that of Diptericin by LPS was not affected (Fig. 8A). Of note, the down-regulation of Ref(2)P by RNAi promotes the release of DaPKC from a Triton-soluble membrane fraction (Fig. 8B), consistent with previously published evidence on the localization of p62 in mammalian cells (28).

Ref(2)P interacts with aPKC in vivo. In the next series of experiments, we determined whether there is a physical interaction between Ref(2)P and the aPKCs as has been demonstrated for p62 (29). Thus, 293 cells were transfected with a tagged version of the Ref(2)P expression plasmid, after which cell extracts were immunoprecipitated with either an irrelevant antibody or an anti-aPKC antibody to precipitate the endogenous aPKCs, and the associated Ref(2)P was determined with the corresponding anti-tag antibody. Interestingly, there is a clear association of aPKC and Ref(2)P in vivo (Fig. 9A). When the interaction between Ref(2)P and αPKC or εPKC was investigated in similar cotransfection experiments, it was clear that Ref(2)P, like p62, was unable to bind these PKC isotypes (data not shown). Of note, the binding of Ref(2)P to DaPKC in S2 cells was also detected (Fig. 9B).

Ref(2)P interacts with DTRAF2 in vivo. The physical and functional interaction between p62 and TRAF6 in mammalian cells is well established (29). Therefore, we next determined whether there is a physical and functional interaction between Ref(2)P and DTRAF2, the *Drosophila* TRAF6 homologue. Thus, 293 cells were transfected with a tagged version of the Ref(2)P expression plasmid with or without a tagged DTRAF2 expression vector, after which DTRAF2 was immunoprecipitated and the associated Ref(2)P was determined by immunoblotting with the corresponding anti-tag antibody. Results shown in Fig. 9C clearly demonstrate the in vivo association of Ref(2)P with DTRAF2. In order to investigate whether there is a functional interaction between both proteins, we sought to determine whether Ref(2)P cooperated with DTRAF2 to activate the Drosomycin promoter. Thus, S2 cells were first transfected with the Drosomycin promoter reporter construct as described above, along with a plasmid control or increasing amounts of a DTRAF2 expression plasmid. Results shown in Fig. 10A demonstrate that the expression of DTRAF2, like that of Ref(2)P (Fig. 6), is sufficient to activate the Drosomycin promoter, in agreement with previously reported results (32). More importantly, the ability of increasing concentrations of Ref(2)P to activate the Drosomycin promoter is synergistically enhanced by the expression of low levels of DTRAF2, which alone do not significantly activate this parameter (Fig. 10B). Collectively, these data indicate that there is a physical and

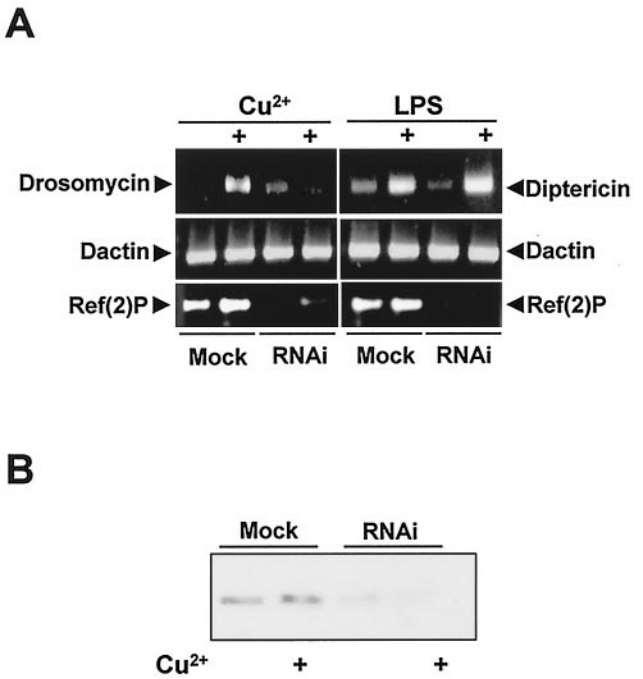

FIG. 8. Ref(2)P is essential for Drosomycin transcription. (A) S2tpll cells were transfected with Ref(2)P dsRNA (RNAi) or were not transfected (Mock), after which they were stimulated with either Cu^{2+} (left panel) or LPS (right panel), and the levels of transcriptional activation of Drosomycin (left panel) and Diptericin (right panel) were determined by RT-PCR. The levels of Ref(2)P RNA were determined in parallel cell extracts by RT-PCR. (B) S2tpll cells were transfected or not transfected with Ref(2)P dsRNA, after which they were stimulated or not stimulated with Cu^{2+}, and the localization of DaPKC in Triton-soluble membrane fractions was determined by immunoblot analysis. The results shown are representative of results from at least two other experiments.

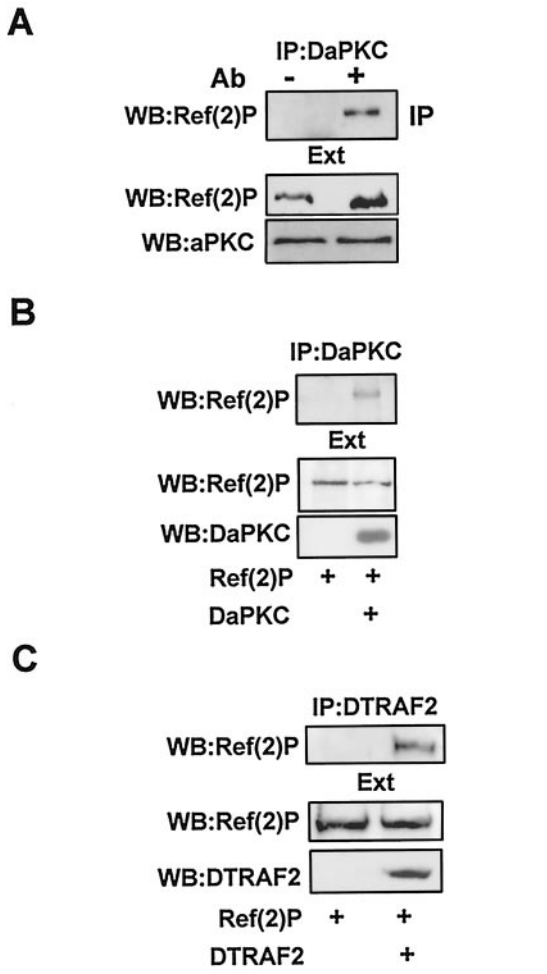

FIG. 9. Ref(2)P interacts with aPKC in vivo. (A) Cultures of 293 cells were transfected with either an empty plasmid or an HA-tagged Ref(2)P expression vector, after which cell extracts (Ext) were immunoprecipitated with an irrelevant (−) or an anti-aPKC (+) antibody (Ab) and the immunoprecipitates (IP) were analyzed with an anti-HA antibody. (B) S2 cells were transfected with a Myc-tagged Ref(2)P expression plasmid along with either an empty vector or a DaPKC expression plasmid. Cell extracts were immunoprecipitated with an anti-aPKC antibody, and the immunoprecipitates were analyzed with an anti-Myc antibody. (C) Cultures of 293 cells were transfected with a Myc-tagged Ref(2)P expression plasmid along with either an empty vector or a Flag-tagged DTRAF2 expression plasmid. Cell extracts were immunoprecipitated with an anti-Flag antibody, and the immunoprecipitates were analyzed with an anti-Myc antibody. Aliquots corresponding to 1/10 of the amount of extracts (Ext) used for the immunoprecipitation were loaded into the gels and analyzed by immunoblotting with the corresponding antibodies. The results shown are representative of results from at least two other experiments. WB, Western blot.

functional interaction between both proteins, as has been reported for TRAF6 and p62 in mammalian cells.

Recombinant ζPKC phosphorylates Dif in vitro. We have previously shown that mammalian ζPKC is able to directly phosphorylate RelA in vitro (20). In order to address whether the *Drosophila* homologues of RelA can also be directly targeted by this kinase, recombinant bacterially expressed Dif was incubated either in the absence or in the presence of recombinant pure ζPKC. The data shown in Fig. 11 demonstrate that ζPKC is able to directly phosphorylate Dif in vitro.

DISCUSSION

The involvement of the aPKCs in NF-κB activation has been extensively documented in a number of studies that used a large variety of experimental strategies (24). Most recently, the generation of a mouse with the ζPKC isoform knocked out has demonstrated that this particular PKC isotype is required for NF-κB-dependent transcriptional activity (20). Thus, in embryonic fibroblasts from $\zeta PKC^{-/-}$ cells, the activation of a κB-dependent reporter as well as the induction of κB-dependent transcripts was, although not completely inhibited, seriously impaired (20). However, the activation of the IKK complex or the nuclear translocation of NF-κB was not affected by the lack of ζPKC in that cell system, indicating that ζPKC, like T2K or GSK-3β (2, 11, 43), appears to act, at least in embryonic fibroblasts, in the control of κB-dependent transcription at a level that is downstream of the translocation of NF-κB. However, in other tissues, such as lung, in which ζPKC levels are much higher than in fibroblasts, the lack of ζPKC also inhibits IKK activation and the nuclear translocation of NF-κB (20). Therefore, it seems that in cells in which ζPKC levels are very

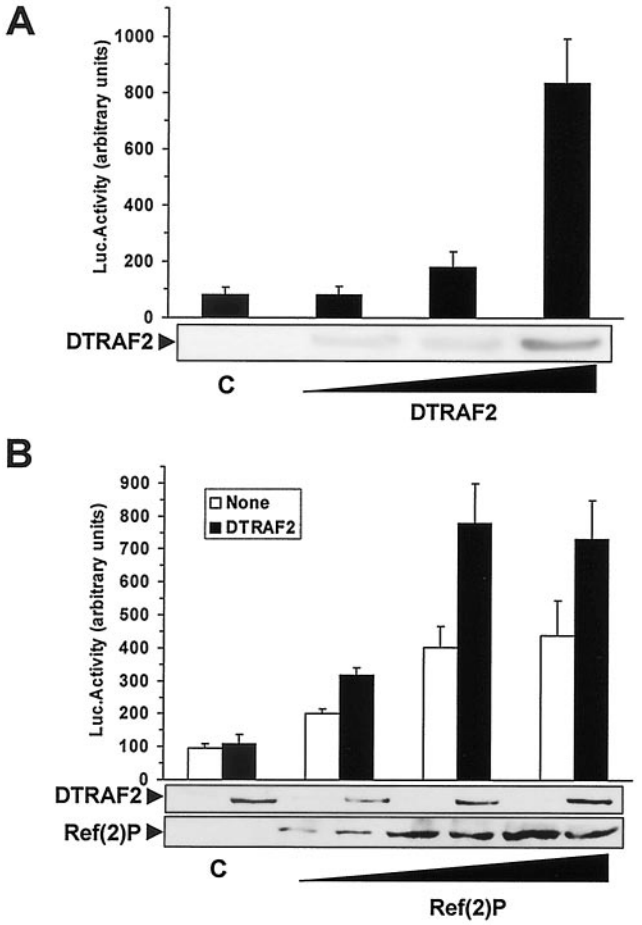

FIG. 10. . Ref(2)P and DTRAF2 cooperate to activate the Drosomycin promoter. S2 cells were transfected with the Drosomycin luciferase reporter as described above, along with increasing amounts (1, 3, and 10 μg) of DTRAF2. (A) In addition, S2 cells were transfected with 1 μg of DTRAF2 expression vector either alone or with increasing amounts (1, 2, and 5 μg) of Ref(2)P expression plasmid (B). Results are the means ± SD of results from three independent experiments with duplicate incubations. Representative immunoblots for Ref(2)P and DTRAF2 performed with the corresponding anti-tag antibodies are shown below the graph.

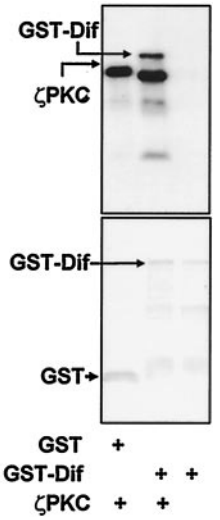

FIG. 11. ζPKC phosphorylates Dif in vitro. Recombinant pure GST-Dif or GST was incubated with pure recombinant ζPKC, and the phosphorylation was determined as described in Materials and Methods. The results shown are representative of results from two other experiments.

low, this PKC isoform plays an essential role in NF-κB-dependent transcriptional activation that cannot be compensated for by λ/ιPKC, which is ubiquitously and abundantly expressed (20); in cells where ζPKC levels are higher, it may function upstream of the IKK complex, possibly as an IKK kinase (19). This is reminiscent of, for example, the cell type-dependent role played by IKKα in NF-κB signaling. Thus, IKKα-deficient fibroblasts show a nearly intact IKK activity in response to tumor necrosis factor alpha (13, 17, 21) but have dramatically impaired activation of κB-dependent transcription (35) due to a lack of p65 activation. However, in mammary epithelial cells (3), IKKα is critical for IκB degradation and NF-κB activation in response to RANK signaling.

Drosophila appears to be an ideal system in which to determine the primary role of the aPKCs in NF-κB signal transduction because it encodes only one aPKC isoform. According to the data presented here, DaPKC is selectively required for the innate immune Toll-signaling pathway, acting downstream of the translocation of Dorsal and Dif and playing a critical role in the induction of the antimicrobial peptide gene for Drosomycin, which is a typical NF-κB-dependent process. Therefore, it can be argued that the primary role of the aPKCs, particularly that of ζPKC in higher eukaryotic cells, is to somehow control the transcriptional activity of NF-κB through a still not completely understood mechanism that most likely involves the direct phosphorylation of RelA (20) and Dif (this study). Interestingly, in *Drosophila* it is well documented that the phosphorylation of Dorsal is required not only for its transcriptional activity but also for its nuclear translocation (1, 8). We did not observe, in the DaPKC-depleted cells, a strong inhibition of Dorsal or Dif nuclear translocation, which suggests that the role of DaPKC is independent of the previously characterized role for Dorsal phosphorylation in regulating nuclear translocation. Based on experiments in mammalian systems, which demonstrate that p65 transcriptional activity must be stimulated by phosphorylation (31), it is possible that the residues that control the transcriptional activities of both Dorsal and Dif are different from those controlling the nuclear import of the protein. It is also possible that DaPKC-mediated phosphorylation has a subtle, yet important, role in the nuclear translocation of Dif and/or Dorsal. Future studies will address this important issue.

The data presented here also demonstrate that Ref(2)P is most likely the functional homologue of p62 in *Drosophila* (Fig. 12). We show that like p62, Ref(2)P interacts physically with the aPKCs. Therefore, it appears that the p62-aPKC signaling module, like the Par/aPKC complex, is highly conserved. Importantly, we also demonstrate a functional role of Ref(2)P in Toll signaling. Thus, the ectopic expression of Ref(2)P is capable by itself of activating the Drosomycin promoter. More interestingly, its depletion severely impairs the Toll pathway (Drosomycin induction) but not the LPS pathway (Attacin

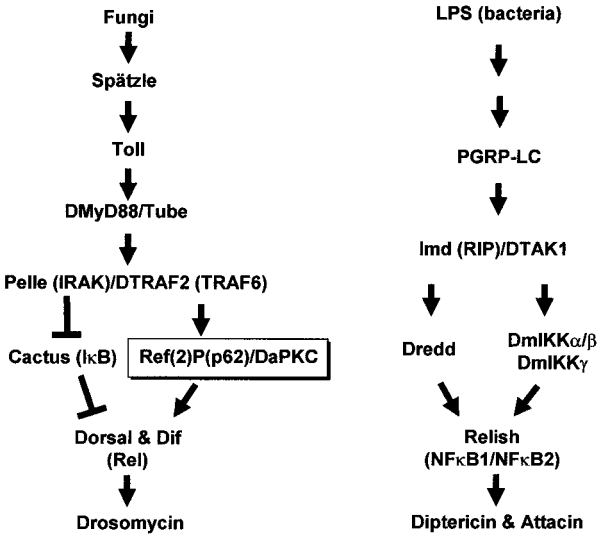

FIG. 12. Innate immunity pathways in *Drosophila*. Triggering the antifungal pathway leads to the activation of Drosomycin transcription. This cascade is initiated when Spätzle binds Toll, recruiting the adapters DMyD88, Pelle, Tube, and possibly DTRAF2. The latter interacts with Ref(2)P, which also binds DaPKC. Both Ref(2)P and DaPKC are essential for Toll-induced Drosomocyn expression, but they are not required for Cactus degradation. Instead, we propose that they stimulate Dif and/or Dorsal transcriptional activity. A parallel pathway is triggered by gram-negative bacteria, which leads to the activation of Diptericin and Attacin transcription. This cascade signals through the receptor peptidoglycan recognition protein (PGRP)-LC and the adapter Imd and activates a *Drosophila* IKK complex. The *D. melanogaster* IKK complex regulates Relish endoproteolytic activation, via the caspase Dredd, and thus controls Diptericin expression. In parentheses are shown the names of the mammalian homologues of Pelle, DTRAF2, Ref(2)P, Dorsal, Dif, Imd, and Relish.

induction). Thus, the Ref(2)P/DaPKC complex is critical for Toll signaling.

The results presented here also demonstrate that, similar to the p62-TRAF6 connection in mammals, Ref(2)P and DTRAF2 physically and functionally interact. Together with the results demonstrating that DaPKC and Ref(2)P are essential for a downstream event in the Toll-signaling pathway, this suggests that a putative Ref(2)P/DaPKC/DTRAF2 complex might function in the signal-induced stimulation of Dif or Dorsal transcriptional activity. In this regard, it is noteworthy that recent results suggest that TRAF6, in addition to its role in IKK recruitment and activation, may also be involved in the control of RelA transcriptional activity (15). However, the role of DTRAF2 in Toll signaling requires further investigation, as the effect of inhibiting (or mutating) DTRAF2 has not yet been reported. Further studies will also address the precise mechanism whereby DaPKC controls the Toll pathway. The data presented here clearly establish the conserved role of the homologue of the p62/aPKC cassette in NF-κB signaling in *Drosophila*.

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