A heterotrimeric death domain complex in Toll signaling

Huaiyu Sun, Benjamin N. Bristow, Guowei Qu, and Steven A. Wasserman†

Center for Molecular Genetics, Section of Cell and Developmental Biology, Division of Biology, University of California at San Diego, La Jolla, CA 92093

Edited by Michael S. Levine, University of California, Berkeley, CA, and approved August 15, 2002 (received for review July 3, 2002)

Signaling from the transmembrane receptor Toll to Rel-related transcription factors regulates dorsoventral patterning of the *Drosophila* embryo, as well as larval and adult immunity. To identify additional pathway components, we have used double-stranded RNA interference to investigate *Drosophila* counterparts of genes that regulate the mammalian Rel family member NF-κB. Experiments in cultured cells reveal that the fly orthologue of the adaptor protein MyD88 is essential for signal transduction from Toll to a second adaptor protein, Tube. By using coimmunoprecipitation studies, we find a heterotrimeric association of the death domains of MyD88, Tube, and the protein kinase Pelle. Site-directed mutational analyses of interaction sites defined by crystallographic studies demonstrate that Tube recruits MyD88 and Pelle into the heterotrimer by two distinct binding surfaces on the Tube death domain. Furthermore, functional assays confirm that the formation of this heterotrimer is critical for signal transduction by the Toll pathway.

The *Drosophila* Toll pathway regulates the establishment of embryonic dorsoventral polarity, as well as the innate immune response against Gram-positive bacteria and fungi (1–3). Toll acts upstream of Tube and Pelle, which in turn are upstream of Cactus, an IκB-like inhibitor. In dorsoventral patterning, this signaling cassette regulates Dorsal, a Rel/NF-κB family member (1). In adult flies, the same Toll pathway acts on both Dorsal and another Rel/NF-κB protein, Dif, to initiate the innate immune response (4, 5).

Tube is a scaffolding protein containing an N-terminal interaction motif belonging to the death domain family, as well as C-terminal Tube repeats that mediate binding to Dorsal (6–8). Pelle is a serine/threonine-specific protein kinase with a death domain N-terminal to its catalytic domain (9, 10).

Although no Tube homologue has been found in mammals, four Pelle homologues, named IL-1 receptor-associated kinases (IRAKs), have been identified: IRAK1, -2, -M, and -4 (11–15). IRAKs function in signaling by a family of Toll-like receptors, as well as the IL-1 receptor (IL-1R), each of which contains a TIR domain, a conserved cytoplasmic signaling motif. An adaptor molecule, MyD88, associates with the C-terminal TIR domain of Toll-like receptors and the IL-1 receptor and with the N-terminal death domain (DD) of IRAKs (16–18).

During the past few years, genomic sequencing has allowed the identification of *Drosophila* genes with mammalian homologues functioning in Toll/IL-1 receptor-signaling pathways. These genes include IKK (a homologue of mammalian IKKα/β), Kenny (a homologue of mammalian IKKγ), IK2 (a homologue of mammalian TBK1/IKKε), MyD88, TAK1, three TRAF loci, and ECSIT (19–28). We have set out to study these genes systematically by using RNA interference (RNAi). RNAi provides a ready means to inactivate a given gene or genes and has facilitated the dissection of *Drosophila* signaling pathways in cultured S2 cells (29). To search for essential components of the Toll pathway, we conducted an RNAi-based screen among these potential *Drosophila* NF-κB regulators. This approach, coupled with genetic and biochemical analyses, has allowed us to dissect the molecular interactions among death domain-containing proteins in the *Drosophila* Toll pathway.

Materials and Methods

Plasmids. All S2 cell expression plasmids are based on the pMT vector (*Drosophila* Expression System, Invitrogen). For bacterial expression, the pGEX-4T vector (Pharmacia) was used. The Toll, Tube, Pelle, and MyD88 constructs in this study were generated from cDNAs by PCR-based subcloning and mutagenesis. TollΔLRR deletes residues 152–800 of Toll; PelleDD encodes residues 1–206; TubeDD encodes residues 1–226; TubeDD* encodes residues 1–253. TubeDD* showed greater resolution from PelleDD in electrophoresis than did TubeDD. We therefore used TubeDD* in place of TubeDD when both Tube and Pelle death domains were expressed with the same epitope tag (as in Fig. 3). The Torso-Tube fusion was subcloned from a construct described (30). Drosomycin-luciferase (pGL3-Drosomycin) and Attacin-luciferase (pGL3-Attacin) were provided by J.-L. Imler (31). IKK (LD21354), Kenny (LD18356), IK2 (SD10041), MyD88 (LD20892), TAK1 (LD42274), ECSIT (RE02264), TRAF1 (LD20987), TRAF2 (GH01161, also known as TRAF6), and TRAF3 (LP08566) are all cDNA clones characterized in the Berkeley *Drosophila* Genome Project (www.fruitfly.org).

S2 Cell Culture and Transfection. S2 cells (American Type Culture Collection) were grown in Schneider's *Drosophila* Medium (GIBCO) supplemented with 10% FBS (HyClone) and 2 mM L-glutamine (Life Technologies, Grand Island, NY). Cells were maintained and transfected as specified for the *Drosophila* Expression System (Invitrogen). For pMT-based vectors, expression was induced by 500 μM $CuSO_4$ 24 h before the cell lysates were prepared. To harvest cells, the culture medium was removed by aspiration and the cells were washed once with PBS before being resuspended in lysis buffer.

RNAi. RNAi in S2 cells was performed essentially as described by Clemens *et al.* (29). Templates (≈700 bp) for double-stranded RNA (dsRNA) synthesis were PCR-amplified from cDNA clones by primers flanked with T7 promoter sequences. dsRNA was then synthesized with the T7 *in vitro* transcription kit (MegaScript, Ambion, Austin, TX). For reporter assays, S2 cells were transfected with 0.4 μg of appropriate reporter plasmid plus 0.4 μg of pAc-LacZ (*Drosophila* Expression System, Invitrogen) in a 10-cm dish 24 h before RNAi. To treat cells with dsRNA, S2 cells were washed once with complete Schneider's medium and then resuspended in serum-free medium to a density of 10^6 cells per milliliter. One milliliter of cell suspension was seeded into each well of a six-well plate and mixed thoroughly with 15 μg of dsRNA. After 45 min, 2 ml of complete medium was added. Cells were harvested 48 h after the addition of dsRNA.

Reporter Assay. For the Drosomycin-luciferase reporter assay, cell lysates were prepared with Reporter Lysis Buffer (Promega)

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: RNAi, RNA interference; dsRNA, double-stranded RNA; IRAK, IL-1 receptor-associated kinase; LPS, lipopolysaccharide.

†To whom reprint requests should be addressed. E-mail: stevenw@ucsd.edu.

DEVELOPMENTAL BIOLOGY

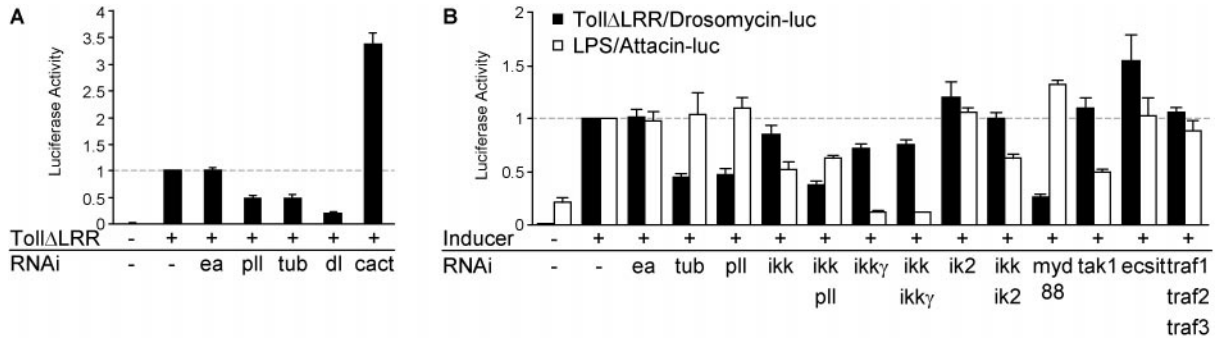

Fig. 1. Identification of Toll pathway molecules by RNAi. (*A*) The *Drosophila* Toll pathway in S2 cells is susceptible to RNAi. S2 cells bearing a copper-inducible TollΔLRR (S2/TollΔLRR) were first transfected with pGL3-Drosomycin and pAc-LacZ, then treated with dsRNA for Easter (ea), Pelle (pll), Tube (tub), Dorsal (dl), or Cactus (cact) followed by induction of TollΔLRR expression. Luciferase activity was measured as described in *Materials and Methods*; error bars indicate standard deviation. (*B*) MyD88 is an essential component of the *Drosophila* Toll pathway. RNAi against Easter, Tube, Pelle, IKK, IKK and Pelle combined, IKKγ, IKKγ and IKK combined, IK2, IK2 and IKK combined, MyD88, TAK1, ECSIT, or TRAF1, 2, and 3 combined were conducted either in S2/TollΔLRR cells as described in *A* (filled bars), or in S2 cells transfected with pGL3-Attacin and pAc-LacZ and subjected to LPS stimulation after RNAi (open bars). Inducer refers to $CuSO_4$ for filled bars and to LPS for open bars.

24 h after the $CuSO_4$ induction of appropriate genes. For the Attacin-luciferase reporter assay, the cells were incubated with 10 μg/ml of lipopolysaccharide (LPS) (Sigma, *Escherichia coli* serotype 026:B6) for 4 h before the cell lysate preparation. Luciferase activity was measured with the Luciferase Assay System kit (Promega) and β-gal activity was measured with the Galacto-Light kit (Tropix, Bedford, MA). Luciferase activity was first normalized with β-gal activity and then normalized against the $CuSO_4$/LPS-induced activity in the absence of any RNAi treatment. Each data point shown in *Results* is the mean ± SD of at least three independent assays.

Anti-MyD88 Antiserum. Full-length *Drosophila* MyD88 was expressed as a GST-fusion protein in *E. coli* BL21 and purified by PAGE. The excised gel slice containing GST-MyD88 was lyophilized, pulverized, and mixed with Freund's incomplete adjuvant for immunization into rabbits. Western blotting with this antibody shows that the exogenous MyD88 accounts for greater than 90% of total MyD88 protein in the whole cell lysate of S2 cells stably transfected with a V5 epitope-tagged MyD88.

Immunoprecipitation. For immunoprecipitation, cell lysates were prepared with S2 cell lysis buffer (50 mM Tris, pH 7.5/150 mM NaCl/1% Nonidet P-40) supplemented with protease inhibitor mixture (Roche Diagnostics) on ice. Six hundred microliters of cleared lysate was prepared from two 35-mm plates of transfected cells. Cell lysates were first incubated for 1 h at 4°C with 1 μg of 9E10 monoclonal antibody (Santa Cruz; for anti-myc immunoprecipitation) or 2 μl of anti-MyD88 antiserum (for anti-MyD88 immunoprecipitation) and then with 10 μl (bed-volume) of protein A/G agarose beads (Santa Cruz). After 3 h, the agarose beads were washed with S2 cell lysis buffer and resuspended in 40 μl of SDS/PAGE sample buffer.

Results

MyD88 Is Essential for *Drosophila* Toll Signaling. To investigate the mechanism of Toll signaling, we used a reporter assay in conjunction with RNAi in cultured *Drosophila* cells. A constitutively active form of Toll, TollΔLRR (32), was stably expressed in S2 cells under the control of a metallothionein promoter, such that the addition of $CuSO_4$ to the cell culture medium initiates Toll signal transduction. To assay signal transduction downstream of Toll, we transiently transfected these S2 cells with a Drosomycin-luciferase construct (31). Expression of TollΔLRR consistently induced a significant activation (≈100 fold) of the Drosomycin reporter (Fig. 1).

To confirm the efficacy of RNAi in these cells, we generated dsRNA for several genes known to function in the *Drosophila* Toll pathway (Fig. 1*A*). RNAi against Pelle, Tube, or Dorsal significantly inhibited the activation of the Drosomycin reporter, with the effect of Dorsal RNAi relatively stronger than that of Pelle or Tube RNAi. In contrast, RNAi against Cactus dramatically enhanced the activation of the Toll pathway. These observations are consistent with the fact that Pelle, Tube, and Dorsal promote Toll signaling, whereas Cactus plays an inhibitory role in the pathway. In this and all subsequent experiments, Easter RNAi served as a negative control for any nonspecific effect of dsRNA, because Easter acts upstream, and not downstream, of Toll.

Next, we performed RNAi-based screening against fly counterparts of mammalian Toll and tumor necrosis factor pathway components, specifically *Drosophila* IKK, IKKγ (Kenny), IK2, MyD88, TAK1, ECSIT, TRAF1, TRAF2, and TRAF3 (Fig. 1*B*). We eliminated expression of each of these genes individually by RNAi in S2 cells and assayed the effect on Toll signaling (Fig. 1*B*, filled bars). We also conducted RNAi against combinations of genes, in particular IKK and Pelle; IKK and IKKγ; IKK and IK2; and TRAF1, 2, and 3. To determine whether requirements were specific to the Toll pathway, we conducted the same panel of RNAi analysis in S2 cells treated with LPS. An Attacin-luciferase reporter (31) was used to indicate LPS-mediated activation of the response pathway for Gram-negative bacteria (Fig. 1*B*, open bars).

When we compared the effects of RNAi on the Toll and LPS pathways, we found that *Drosophila* MyD88, like Tube and Pelle, was required for activation of the Drosomycin, but not the Attacin, reporter; *Drosophila* MyD88 is thus essential for Toll signaling, as reported (19, 20). In contrast, we found a requirement for TAK1 only in the LPS pathway and found no essential role for fly IK2, ECSIT, or TRAF 1, 2, and 3 in either Toll or LPS signaling. Inactivating IKK and IKKγ affected both types of signaling, with the LPS pathway being more severely inhibited than the Toll pathway. These results are consistent with the fact that inactivating IKK in flies disrupts Toll-dependent axis formation in a small fraction of embryos (20), although neither IKK nor IKKγ is strictly required for Toll signal transduction (19, 33).

MyD88, Tube, and Pelle Form a Linear Signaling Hierarchy. It is known that Tube acts downstream of Toll and upstream of Pelle in signal transduction (30, 34, 35). To place *Drosophila* MyD88 in this pathway precisely, we analyzed the epistatic relationship

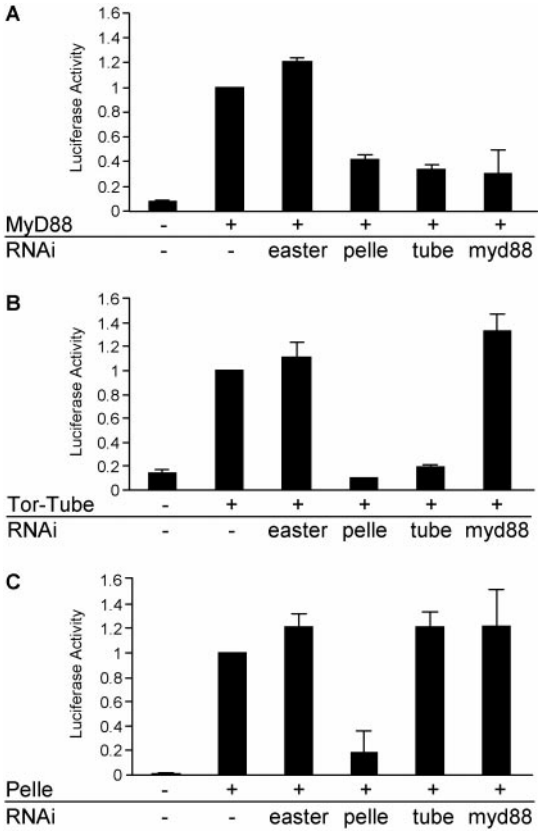

Fig. 2. Signaling hierarchy formed by MyD88, Tube, and Pelle. S2 cells carrying copper-inducible (*A*) MyD88, (*B*) Torso-Tube (Tor-Tube), or (*C*) Pelle were transfected with the Drosomycin reporter and pAc-LacZ. Cells were then treated with the indicated panel of dsRNA (easter, pelle, tube, or myd88), and induced to express corresponding proteins with $CuSO_4$. Luciferase activity was determined as described in *Materials and Methods*.

among MyD88, Tube, and Pelle. We first induced expression of wild-type MyD88, which has been shown to activate the Drosomycin reporter (21). RNAi against Pelle, Tube, or MyD88 blocked this MyD88-induced activation (Fig. 2*A*). These results, as well as similar findings in adult flies (22), indicate that MyD88 acts either upstream of or in parallel to Tube.

To dissect the signaling hierarchy further, a constitutively active form of Tube (30, 35) was used (Fig. 2*B*). This Tube-initiated activation of the Drosomycin reporter did not require MyD88, but did require Pelle. Furthermore, Pelle-induced activation of the Drosomycin reporter was diminished only by RNAi against Pelle, but not Tube or MyD88 (Fig. 2*C*). Thus, epistasis analysis defines a linear order of action, with Tube downstream of MyD88 and upstream of Pelle.

Tube Mediates the Association of MyD88 and Pelle. MyD88, Tube, and Pelle each contain a death domain, a motif known to form homotypic interactions (36–39). We have previously shown that Tube and Pelle interact directly by means of their death domains (40). Furthermore, MyD88 has been found to coimmunoprecipitate with Pelle in S2 cells (21, 22). We were therefore interested in the role of binding interactions mediated by death domains in the hierarchy defined by epistasis analysis.

To assay the interaction of MyD88 with either Pelle or Tube, we epitope tagged full-length MyD88, as well as the death domain of Pelle (PelleDD) and a slightly larger Tube death domain peptide (TubeDD*; see *Materials and Methods*). We also generated an antiserum against *Drosophila* MyD88. We then

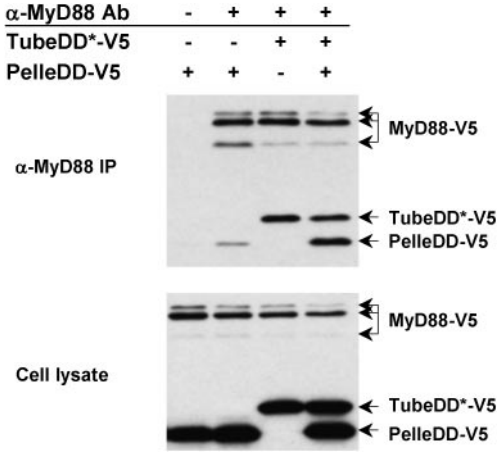

Fig. 3. Tube is required for the interaction between MyD88 and Pelle. A stable cell line expressing MyD88 was transfected with PelleDD, TubeDD*, or PelleDD and TubeDD* combined. Immunoprecipitates were prepared with preimmune serum (−) or anti-MyD88 antiserum (+) as indicated. All three peptides were detected by an antibody against V5 epitope tag in either immunoprecipitates (*Upper*) or whole-cell lysates (*Lower*).

carried out immunoprecipitation experiments, using the α-MyD88 for the precipitation step and α-V5 to detect the tagged peptides (Fig. 3). In pair-wise experiments, we detected substantial interaction between MyD88 and the Tube death domain. (In addition, a reduction occurred in the abundance of a fast migrating MyD88 species, perhaps reflecting a TubeDD-mediated protection from proteolysis). In contrast, only a trace amount of PelleDD coprecipitated with MyD88.

Next, we coexpressed PelleDD, TubeDD, and MyD88 to assay for higher-order complexes. Under such conditions, the amount of MyD88-associated PelleDD was dramatically increased. Indeed, the relative amount of TubeDD and PelleDD coimmunoprecipitated with MyD88 was indistinguishable (Fig. 3). We conclude that Tube forms a stable complex with MyD88 and is also strictly required for the recruitment of Pelle into a complex with MyD88. (Mutational analysis presented below demonstrates that the association of a minor amount of PelleDD with MyD88 in the absence of TubeDD is mediated by endogenous Tube protein.)

Heterotrimer Formation Involves Two Binding Surfaces on the Tube Death Domain. We envisioned two alternative models for the role of Tube in complex formation. In one, the interaction of Pelle and Tube is essential for Pelle to join the MyD88 complex. In the alternative model, Pelle can stably associate with MyD88, provided MyD88 is bound by Tube. To discriminate between these two models, we used interaction surface mutations we had generated in characterizing a dimer between the Tube and Pelle death domains.

The crystal structure of the complex formed by the death domains of Tube and Pelle suggested that residue E50 in Tube and R35 in Pelle form a salt bridge that is critical for dimer formation (Fig. 4*A*). By using an RNA injection bioassay, we previously demonstrated that mutation of residue 50 in Tube to lysine (E50K mutation) abolished Tube function in Toll signaling (40). We were therefore surprised to find that the E50K mutation had no discernible effect on the binding of the Tube death domain to MyD88 (Fig. 4*B*).

Although Tube E50K had an apparently wild-type interaction with MyD88, this mutation blocked the binding of Tube to Pelle in our coimmunoprecipitation assay (Fig. 4*C*). Furthermore, a mutational change in Pelle (Pelle R35E) that is predicted to reconstitute the salt bridge fully restored the Tube–Pelle inter-

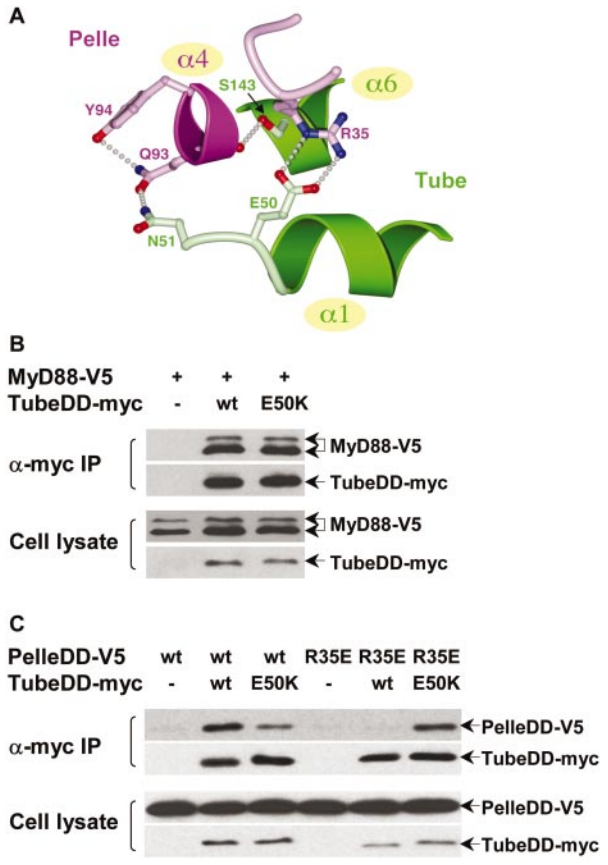

Fig. 4. Tube interacts with MyD88 and Pelle through distinct physical contacts. (*A*) Pelle–Tube interaction requires an ion pair formed by Pelle R35 and Tube E50. (*B*) Comparison of wild-type (wt) TubeDD and TubeDD E50K mutant in their interaction with MyD88. Transiently transfected MyD88 and TubeDD are detected with the anti-V5 and anti-myc antibodies, respectively, in either anti-myc immunoprecipitates (IP) (*Upper*) or whole-cell lysates (*Lower*). Mutant forms are as indicated. (*C*) Coimmunoprecipitation of PelleDD and TubeDD. Similar to *B*, except MyD88 was replaced by PelleDD or PelleDD R35E.

action (Fig. 4*C*), just as these compensatory mutations in Tube and Pelle together allowed signaling in embryos (40). Thus, at least two types of death domain contacts are in the Toll signaling complex: one between Tube and Pelle that involves Tube E50 and a second between Tube and MyD88 that is E50-independent.

To determine whether binding to Tube is essential for Pelle recruitment into the MyD88 complex, we took further advantage of the compensatory mutations in Tube and Pelle. In cells coexpressing TubeDD and PelleDD, the association of PelleDD with MyD88 was greatly inhibited by either individual mutation, Pelle R35E or Tube E50K, that blocks the Tube–Pelle interaction (Fig. 5*A*, compare lanes 7–9, as well as lanes 2 and 3). Remarkably, the simultaneous presence of these compensatory mutations restored the recruitment of PelleDD to the MyD88 complex (Fig. 5*A*, lanes 7 and 10). We therefore conclude that Pelle must bind directly to Tube to join the MyD88 complex (Fig. 5*B*).

The Heterotrimer of MyD88, Tube, and Pelle Mediates Toll Signaling. On the basis of our model of the heterotrimeric death domain complex (Fig. 5*B*), we predicted that expression of the wild-type death domain of either Tube or Pelle might disrupt formation of an endogenous trimeric complex and thereby interfere with the normal function of the Toll pathway. Moreover, we expected distinct outcomes for expression of mutant forms of the Tube and Pelle death domains. The E50K mutant of TubeDD, although incapable of interacting with Pelle, nevertheless binds to MyD88 and hence might interfere with the formation of the complex of MyD88, Tube, and Pelle. By the same logic, expressing the R35E mutant of Pelle, which cannot stably interact with Tube, and hence the trimeric complex, might not interfere with signaling.

To test these hypotheses, we assayed the effect of expressing Tube and Pelle death domains in the context of an active Toll pathway (Fig. 6). Wild-type and E50K TubeDD each significantly blocked TollΔLRR-induced activation of the Drosomycin reporter, as did wild-type PelleDD. However, the R35E mutant of PelleDD, expressed at the same level as its wild-type counterpart, had no discernible effect on Drosomycin activation.

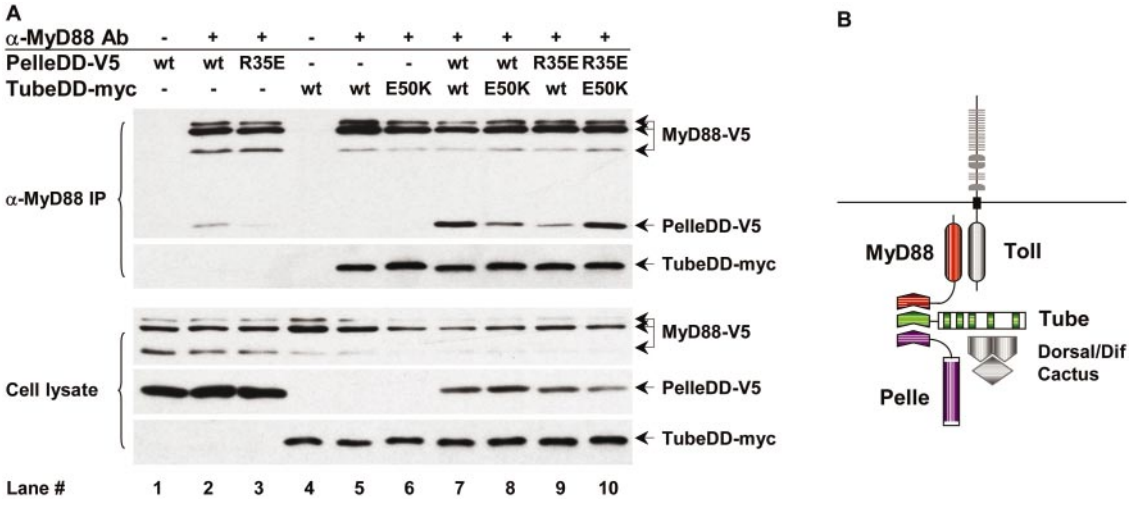

Fig. 5. MyD88, Tube, and Pelle form a trimeric complex. (*A*) An intact Tube:Pelle interaction is critical for the formation of the MyD88/Tube/Pelle complex. S2 cells stably expressing MyD88 were transfected with PelleDD, PelleDD R35E, TubeDD, or TubeDD E50K, or PelleDD and TubeDD together in various combinations as indicated. Immunoprecipitates were prepared with preimmune serum (−) or anti-MyD88 serum (+). MyD88, PelleDD, and PelleDD R35E were detected with an anti-V5 antibody; TubeDD and TubeDD E50K were detected with an anti-myc antibody. (*B*) A model for the signal transduction of the Toll pathway mediated by physical interactions of death domain-containing proteins MyD88, Tube, and Pelle. The Tube-mediated complex formation involves two distinct binding surfaces on Tube death domain, which allow simultaneous association of MyD88 and Pelle. Direct binding of MyD88 to Toll and of both Tube and Pelle to Cactus-bound Dorsal would result in a complex facilitating efficient signal transduction from Toll to Dorsal. wt, wild-type.

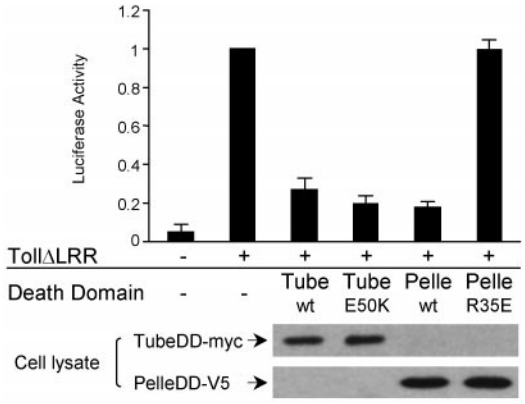

Fig. 6. The dominant negative effect of Tube and Pelle death domains. S2 cells stably expressing a copper-inducible TollΔLRR were transiently transfected with pGL3-Drosomycin and pAc-LacZ, as well as empty vector (−) or the death domain of Tube, Tube E50K, Pelle, or Pelle R35E, as indicated. Luciferase activity was measured 48 h after transfection as described in *Materials and Methods*. The expression of the death domains in the cell lysates for luciferase assay was detected with anti-myc or anti-V5 antibody, as shown (*Bottom*). wt, wild type.

These results thus confirm the predictions of our model for heterotrimer formation and demonstrate that formation of the trimeric MyD88, Tube, and Pelle complex is a critical step in Toll signaling.

Discussion

The death domain was originally identified as a protein module transducing apoptotic signals (41, 42). It has been found, for example, that death domain mediated interactions between Fas and FADD or between tumor necrosis factor receptor and TRADD provide the basis for assembling the death-inducing signaling complex (36, 37). The death effector domain and caspase recruitment domain also form homotypic interactions involved in apoptotic signaling and are structurally similar to a death domain. These motifs, together with the death domain, comprise the death domain superfamily (43).

Our experimental data demonstrate that PelleDD and MyD88 are found in the same complex when each is physically associated with TubeDD (Fig. 5*B*). The association of three different death domains has also been implied by studies on the tumor necrosis factor receptor complex, in which TRADD was found to facilitate the recruitment of FADD or RIP to tumor necrosis factor receptor (44, 45). Here, we have probed the nature of such a complex and have found that MyD88, Pelle, and Tube form a heterotrimer, with the TubeDD interacting with MyD88 and PelleDD by distinct binding surfaces.

Recently, molecular modeling based on available structural data suggested that the homotypic interaction among death domain superfamily modules could be multivalent (43, 46). Higher-order multimers, such as a heterohexamer, can be modeled by docking the death domains of Fas and FADD together. Furthermore, the structural plasticity observed in the PelleDD:TubeDD dimer (40) hypothetically allows it to accept a third death domain into a three-fold symmetric structure (43). Whether the death domains of MyD88, Tube, and Pelle can form such a structure, as opposed to a linear array (Fig. 5*B*), awaits biophysical characterization of this trimeric complex. We note, however, that our assays do not provide evidence for any physical interaction between Pelle and MyD88. In addition, the fact that the PelleDD R35E mutant failed to dominantly interfere with Toll signaling in a functional assay (Fig. 6) argues against the possibility of such a direct contact between Pelle and MyD88.

Because MyD88 binds to Toll through interaction between TIR domains on both proteins (16, 18), we envision that MyD88 connects both Tube and Pelle to Toll. Toll-initiated aggregation of these signaling molecules could trigger Pelle activation. Such a model is consistent with epistasis analyses presented here and elsewhere indicating a linear order of action for Toll, MyD88, Tube, and Pelle in primary signaling (22, 30, 34, 35). Furthermore, because Dorsal binds directly to Pelle, Tube, and Cactus (7, 8, 47), it is conceivable that the entire signaling cassette exists, at least transiently, in a single complex (Fig. 5*B*). As suggested by both biochemical and biological assays (48, 49), Pelle-catalyzed phosphorylation may then lead to both Dorsal nuclear transport and complex dissociation.

In mammalian signaling pathways initiated by either Toll-like receptors or IL-1 receptors, MyD88 associates with IRAK (16–18). Because our study shows that a third death domain is required to mediate the interaction between MyD88 and Pelle in *Drosophila*, does a parallel exist in mammals? Although no known Tube orthologue exists in mammals, multiple IRAKs are present. We speculate, therefore, that two or more IRAKs may participate in one protein complex, with the death domain of one IRAK bridging the interaction of another with MyD88. In this way, a particular IRAK isoform might act together with MyD88 to regulate the activity of a second IRAK through the oligomerization of death domains, resulting in isoform-specific biological functions (14, 15).

We thank Dr. Jean-Luc Imler (Institut de Biologie Moléculaire) for providing the luciferase reporter constructs, Lara-Miya Philips (Howard Hughes Medical Institute/University of California at San Diego) for providing S2 cells, and the Rosenfeld lab (Howard Hughes Medical Institute/University of California at San Diego) for providing access to their luminometer. We also thank Julie Shih and Daniel Chiem for technical assistance and Par Towb for stimulating discussions. We are grateful to T. Xiao and S. Sprang (Howard Hughes Medical Institute/University of Texas Southwestern Medical Center, Dallas) for the illustration in Fig. 4*A*. The research described here was supported by National Institutes of Health Grant 5R01GM50545.

1. Belvin, M. P. & Anderson, K. V. (1996) *Annu. Rev. Cell Dev. Biol.* **12,** 393–416.
2. Hoffmann, J. A. & Reichhart, J. M. (2002) *Nat. Immunol.* **3,** 121–126.
3. Wasserman, S. A. (2000) *Curr. Opin. Genet. Dev.* **10,** 497–502.
4. Rutschmann, S., Jung, A. C., Hetru, C., Reichhart, J. M., Hoffmann, J. A. & Ferrandon, D. (2000) *Immunity* **12,** 569–580.
5. Silverman, N. & Maniatis, T. (2001) *Genes Dev.* **15,** 2321–2342.
6. Letsou, A., Alexander, S., Orth, K. & Wasserman, S. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 810–814.
7. Edwards, D. N., Towb, P. & Wasserman, S. A. (1997) *Development (Cambridge, U.K.)* **124,** 3855–3864.
8. Yang, J. & Steward, R. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 14524–14529.
9. Shelton, C. A. & Wasserman, S. A. (1993) *Cell* **72,** 515–525.
10. Feinstein, E., Kimchi, A., Wallach, D., Boldin, M. & Varfolomeev, E. (1995) *Trends Biochem. Sci.* **20,** 342–344.
11. Cao, Z., Henzel, W. J. & Gao, X. (1996) *Science* **271,** 1128–1131.
12. Muzio, M., Ni, J., Feng, P. & Dixit, V. M. (1997) *Science* **278,** 1612–1615.
13. Wesche, H., Gao, X., Li, X., Kirschning, C. J., Stark, G. R. & Cao, Z. (1999) *J. Biol. Chem.* **274,** 19403–19410.
14. Li, S., Strelow, A., Fontana, E. J. & Wesche, H. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 5567–5572.
15. Suzuki, N., Suzuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., *et al.* (2002) *Nature (London)* **416,** 750–756.
16. Wesche, H., Henzel, W. J., Shillinglaw, W., Li, S. & Cao, Z. (1997) *Immunity* **7,** 837–847.
17. Burns, K., Martinon, F., Esslinger, C., Pahl, H., Schneider, P., Bodmer, J. L., Di Marco, F., French, L. & Tschopp, J. (1998) *J. Biol. Chem.* **273,** 12203–12209.
18. Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S. & Janeway, C. A., Jr. (1998) *Mol. Cell* **2,** 253–258.

19. Silverman, N., Zhou, R., Stoven, S., Pandey, N., Hultmark, D. & Maniatis, T. (2000) *Genes Dev.* **14,** 2461–2471.
20. Lu, Y., Wu, L. P. & Anderson, K. V. (2001) *Genes Dev.* **15,** 104–110.
21. Horng, T. & Medzhitov, R. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 12654–12658.
22. Tauszig-Delamasure, S., Bilak, H., Capovilla, M., Hoffmann, J. A. & Imler, J. L. (2002) *Nat. Immunol.* **3,** 91–97.
23. Zapata, J. M., Matsuzawa, S., Godzik, A., Leo, E., Wasserman, S. A. & Reed, J. C. (2000) *J. Biol. Chem.* **275,** 12102–12107.
24. Shen, B., Liu, H., Skolnik, E. Y. & Manley, J. L. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 8596–8601.
25. Liu, H., Su, Y. C., Becker, E., Treisman, J. & Skolnik, E. Y. (1999) *Curr. Biol.* **9,** 101–104.
26. Kopp, E., Medzhitov, R., Carothers, J., Xiao, C., Douglas, I., Janeway, C. A. & Ghosh, S. (1999) *Genes Dev.* **13,** 2059–2071.
27. Grech, A., Quinn, R., Srinivasan, D., Badoux, X. & Brink, R. (2000) *Mol. Immunol.* **37,** 721–734.
28. Vidal, S., Khush, R. S., Leulier, F., Tzou, P., Nakamura, M. & Lemaitre, B. (2001) *Genes Dev.* **15,** 1900–1912.
29. Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A. & Dixon, J. E. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 6499–6503.
30. Galindo, R. L., Edwards, D. N., Gillespie, S. K. & Wasserman, S. A. (1995) *Development (Cambridge, U.K.)* **121,** 2209–2218.
31. Tauszig, S., Jouanguy, E., Hoffmann, J. A. & Imler, J. L. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 10520–10525.
32. Winans, K. A. & Hashimoto, C. (1995) *Mol. Biol. Cell* **6,** 587–596.
33. Rutschmann, S., Jung, A. C., Zhou, R., Silverman, N., Hoffmann, J. A. & Ferrandon, D. (2000) *Nat. Immunol.* **1,** 342–347.
34. Hecht, P. M. & Anderson, K. V. (1993) *Genetics* **135,** 405–417.
35. Grosshans, J., Bergmann, A., Haffter, P. & Nusslein-Volhard, C. (1994) *Nature (London)* **372,** 563–566.
36. Hsu, H., Xiong, J. & Goeddel, D. V. (1995) *Cell* **81,** 495–504.
37. Chinnaiyan, A. M., O'Rourke, K., Tewari, M. & Dixit, V. M. (1995) *Cell* **81,** 505–512.
38. Stanger, B. Z., Leder, P., Lee, T. H., Kim, E. & Seed, B. (1995) *Cell* **81,** 513–523.
39. Cleveland, J. L. & Ihle, J. N. (1995) *Cell* **81,** 479–482.
40. Xiao, T., Towb, P., Wasserman, S. A. & Sprang, S. R. (1999) *Cell* **99,** 545–555.
41. Tartaglia, L. A., Ayres, T. M., Wong, G. H. & Goeddel, D. V. (1993) *Cell* **74,** 845–853.
42. Itoh, N. & Nagata, S. (1993) *J. Biol. Chem.* **268,** 10932–10937.
43. Weber, C. H. & Vincenz, C. (2001) *Trends Biochem. Sci.* **26,** 475–481.
44. Hsu, H., Shu, H. B., Pan, M. G. & Goeddel, D. V. (1996) *Cell* **84,** 299–308.
45. Hsu, H., Huang, J., Shu, H. B., Baichwal, V. & Goeddel, D. V. (1996) *Immunity* **4,** 387–396.
46. Weber, C. H. & Vincenz, C. (2001) *FEBS Lett.* **492,** 171–176.
47. Kidd, S. (1992) *Cell* **71,** 623–635.
48. Shen, B. & Manley, J. L. (1998) *Development (Cambridge, U.K.)* **125,** 4719–4728.
49. Towb, P., Bergmann, A. & Wasserman, S. A. (2001) *Development (Cambridge, U.K.)* **128,** 4729–4736.