

Structural Complementarity of Toll/Interleukin-1 Receptor Domains in Toll-like Receptors and the Adaptors Mal and MyD88*

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The Toll/interleukin 1 receptor (TIR) domain is a region found in the cytoplasmic tails of members of the Toll-like receptor/interleukin-1 receptor superfamily. The domain is essential for signaling and is also found in the adaptor proteins Mal (MyD88 adaptor-like) and MyD88, which function to couple activation of the receptor to downstream signaling components. Experimental structures of two Toll/interleukin 1 receptor domains reveal a α - β -fold similar to that of the bacterial chemotaxis protein CheY, and other evidence suggests that the adaptors can make heterotypic interactions with both the receptors and themselves. Here we show that the purified TIR domains of Mal and MyD88 can form stable heterodimers and also that Mal homodimers and oligomers are dissociated in the presence of ATP. To identify structural features that may contribute to the formation of signaling complexes, we produced models of the TIR domains from human Toll-like receptor 4 (TLR4), Mal, and MyD88. We found that although the overall fold is conserved the electrostatic surface potentials are quite distinct. Docking studies of the models suggest that Mal and MyD88 bind to different regions in TLRs 2 and 4, a finding consistent with a cooperative role of the two adaptors in signaling. Mal and MyD88 are predicted to interact at a third non-overlapping site, suggesting that the receptor and adaptors may form heterotetrameric complexes. The theoretical model of the interactions is supported by experimental data from glutathione S-transferase pull-downs and co-immunoprecipitations. Neither theoretical nor experimental data suggest a direct role for the conserved proline in the BB-loop in the association of TLR4, Mal, and MyD88. Finally we show a sequence relationship between the *Drosophila* protein Tube and Mal that may indicate a functional equivalence of these two adaptors in the *Drosophila* and vertebrate Toll pathways.

In humans, innate immune responses provide the first line of defense against invading bacterial pathogens. Pathogen-associated molecular patterns such as lipopolysaccharide (LPS)¹ from Gram-negative bacteria are sensed by macrophages, and

these cells respond by producing pro-inflammatory cytokines (e.g. interleukin 1), which provoke the acute phase response to infection (for review see Ref. 1). In recent years, it has become clear that the human Toll-like receptors (TLRs) are required to mediate these responses. These molecules are single pass transmembrane receptors, and they are related to *Drosophila* Toll, a protein involved in dorso-ventral patterning and anti-fungal innate immunity in the fly (2–4). *Drosophila* Toll and TLRs all have ectodomains with characteristic blocks of leucine-rich repeats and a cytoplasmic signaling domain of ~200 residues called the Toll/interleukin 1 receptor (TIR) domain. The family of Toll receptors appears to use common components in the post-receptor signaling pathway, resulting in the activation of the transcription factor NF κ B (5, 6).

It is now well established that human TLR4 together with two other membrane proteins CD14 and MD2 are able to sense the presence of LPS and to establish the appropriate signaling response (7). For example, strains of mice that are hyposensitive to LPS either lack or have inactivating mutations in TLR4 (8). Activation of the receptor is thought to involve dimerization followed by recruitment of a post-receptor complex containing the adaptor MyD88 and the protein kinase IRAK (interleukin 1 receptor-associated kinase) (9). MyD88 is a critical component in the pathway and is required by most if not all of the TLRs (10). It is a modular protein with a TIR and protein interaction domain termed the death domain (11). The TIR domain is thought to bind with that of the receptor, and the death domain appears to interact with the death domain found at the N terminus of IRAK.

Although MyD88 is the principal transducer of TLR signaling, the TLR4 pathway appears to have evolved a second adaptor called Mal (MyD88 adaptor-like) or TIRAP (TIR adaptor protein) (12, 13). Similar to MyD88, this protein has a TIR but it lacks a death domain. In *Drosophila*, a clear homologue of MyD88 is involved in the Toll-mediated innate immune responses (14, 15). However, in dorso-ventral patterning, a different adaptor, Tube, is used. Similar to MyD88, this protein has an N-terminal death domain but it lacks an obvious TIR domain (16).

There is now consistent biochemical evidence that activation of TLRs involves the recruitment of specific post-receptor signaling complexes. However, there is still only limited structural information regarding these complexes. In fact, the only structures solved to date are those of the isolated TIRs from TLRs 1 and 2 (17). These molecules have an overall fold with a central five-stranded parallel β -sheet surrounded by five helices. The structures are related to the bacterial chemotaxis protein CheY, confirming a prediction made by Bazan *et al.*, (6, 18). Most of the conserved residues in TIR domains are buried in the core of the fold, but some are solvent-exposed residues

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¹ The abbreviations used are: LPS, lipopolysaccharide; TLR, Toll-like receptor; TIR, Toll/interleukin 1 receptor domain; Mal, MyD88 adaptor-like; HEK, human embryonic kidney; HA, hemagglutinin; GST, glutathione S-transferase; P/H, proline to histidine mutation.

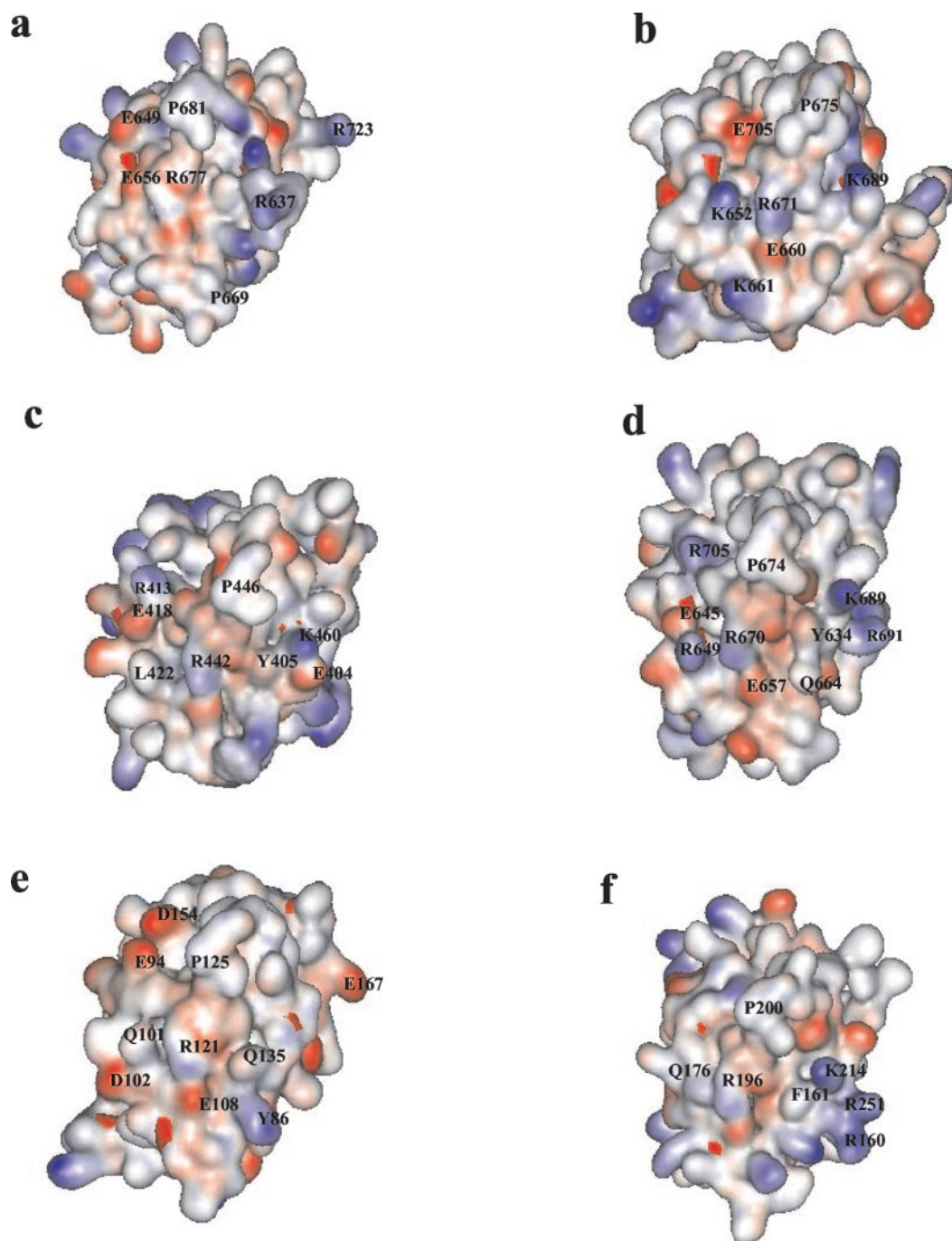


FIG. 1. **Surface charge distribution in TIR domains.** Electrostatic surface potential representations of the S face with *red* indicating areas of negative charge and *blue* indicating areas of positive charge. *a*, TLR2. *b*, TLR1. *c*, interleukin-1RAcP (1 receptor accessory protein). *d*, TLR4. *e*, Mal. *f*, Myd88 TIR-domain models. The structures are projected such that the BB-loop faces the viewer. Residues that contribute to the surface charge are indicated and *numbered* according to a structure based sequence alignment.

located on one face of the protomer. This may form an interaction surface to which MyD88 and Mal can bind in response to signaling. In fact, a loop termed the BB-loop connecting the second β -strand and second helix includes a proline residue that is changed to histidine in mice, which are hyporesponsive to LPS, and the corresponding change also abolishes the ability of TLR2 to interact with MyD88 *in vitro* (8, 17).

In this paper, we have modeled the TIR domains of TLR4, MyD88, and Mal. Experimental data are provided to support results obtained from docking studies, and putative interaction motifs are identified with particular attention being paid to the conserved proline residue found within the BB-loop of the receptor and adaptor TIR domains. These structures suggest

mechanisms by which binding and specificity are achieved by different combinations of TIRs during signaling. We also report sequence similarities between Mal and the *Drosophila* adaptor Tube and the ATP-dependent dissociation of Mal dimers and oligomers.

EXPERIMENTAL PROCEDURES

Modeling Studies—The sequences of target proteins were extracted from the protein sequence data banks. Sequence homologues were retrieved using PSI-BLAST and aligned using ClustalW. The resulting alignment was carefully studied and further adjusted manually using a UNIX-based sequence alignment editor, SEAVIEW. Percentage similarities between homologues were scored using MALFORM.

Homologues with known three-dimensional structures were aligned

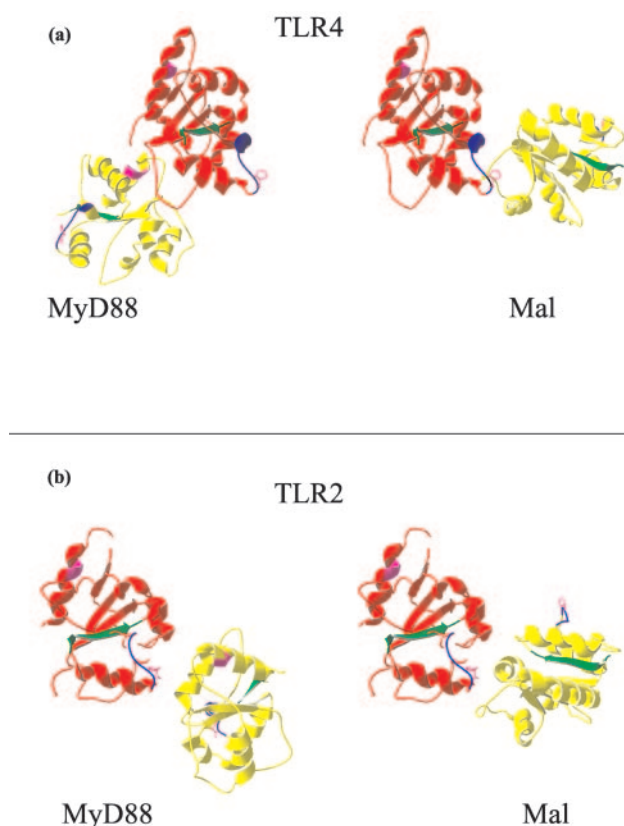


FIG. 2. Model of the docking complexes formed between the TIR domains of human (h) TLR2 and hTLR4 and the TIR domains of Mal and MyD88. Receptor TIR domains are shown in red, and adapter TIR domains are shown in yellow. Structural features representing the conserved boxes of the TIR domains are shown in green (box 1), blue (box 2, BB-loop), and purple (box 3, not present in Mal). The side chain of the semi-conserved proline residue in the BB-loop is colored purple. a, GRAMM docking of the MyD88 (left) and Mal (right) TIR domains to the TIR domain of hTLR4; b, docking of MyD88 (left) and Mal (right) TIR domains to the TIR domain of hTLR2.

using COMPARE (19) or FuGue (20). The JOY annotation used in structure based sequence alignments has been described previously (21, 22). The target sequence was then aligned to the structure based alignment using ClustalX. This alignment was conducted such that gaps or insertions within conserved secondary structure regions are disfavored by constructing a SWISS-PROT style secondary structure mask. MODELLER6a was used to generate 15 models for each protein. Five models with the best combination of low energy and low number of restraint violations were chosen. PROCHECK was used to check the stereochemical quality of models (23, 24). Loop regions with residue geometry problems were further modeled using RAPPER (25, 26). Models were analyzed further using PROSAIL 3.0 or Verify 3D, which use α - and β -based potentials to calculate energy profiles for protein structures. Models of complexes between TLR4 and the adaptor proteins, Mal and MyD88, were constructed using geometry and hydrophobicity complementarity search algorithm in GRAMM. The docking parameters were step grid at 6.8 Å, repulsion potential at 6.5, and intervals of rotation at 10°. One hundred models were generated, and the twenty lowest energy matches were analyzed.

Cell Culture, Plasmids, and Reagents—HEK293 cells were obtained from the Centre for Applied Microbiology & Research (CAMR, Porton Down, Salisbury, Wiltshire, United Kingdom) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units ml^{-1} gentamycin, and 2 mM *l*-glutamine and maintained at 37 °C in a humidified atmosphere of 5% CO_2 . HEK293 cells stably expressing FLAG-tagged TLR-4 were a generous gift from Douglas Golenbock. Cells were seeded at 10^5 ml^{-1} 24 h prior to transfection. The FLAG-tagged TLR4-expressing plasmid was a kind gift from Ruslan Medzhitov (Yale University School of Medicine, New Haven, CT). The MyD88-expressing plasmid was a gift from Marta Muzio (Mario Negri Institute, Milan, Italy). HA-tagged Mal from the pDC304 vector was subcloned into the pCDNA3 expression vector for mammalian

cell-based transfections. For bacterial expression, cDNAs encoding full-length Mal, MyD88, and the TIR domain of TLR4 were amplified by PCR using a spleen library and cloned into the *Bam*HI and *Eco*RI sites of pGEX-4T2 (Amersham Biosciences). Point mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the protocol of the manufacturer. All of the other reagents were obtained from Sigma (Poole, Dorset, United Kingdom) unless otherwise stated.

Co-expression and Purification of Mal and MyD88—Mal and MyD88 full-length expression vectors were co-transformed into *Escherichia coli* BL21 (DE3) and grown in Luria broth. A bacterial cell culture was grown to $A_{600} = 0.6$ under ampicillin and kanamycin selection. Protein expression was induced with 0.7 mM isopropyl-1-thio- β -D-galactopyranoside. Bacterial cells were harvested and resuspended in 50 mM Tris buffer, pH 7.6, 0.15 M NaCl, 5 mM dithiothreitol, 5 mM EDTA, 0.01% Triton X-100, EDTA-free protease inhibitor mixture (Roche Applied Science). Cells were lysed in the French press at 15000 p.s.i., and the soluble/insoluble fractions were separated by centrifugation at $100,000 \times g$. Protein purification was facilitated by affinity purification on glutathione-Sepharose, anion-exchange and size exclusion chromatography (fast protein liquid chromatography).

Immunoprecipitation and GST Pull-down Assays—HEK293 cells were seeded (10^5 ml^{-1}) onto 100-mm dishes 24 h prior to transfection with combinations of plasmids (4 μg of each) as indicated using Gene-juce (Novagen) according to the manufacturer's recommendations. 24–48-h post-transfection cells were washed by the addition of 5 ml of ice-cold phosphate-buffered saline. Cells were lysed on ice (30 min) in buffer containing 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM Na_3VO_4 , and 1 μg of leupeptin ml^{-1} . Immune complexes were immunoprecipitated by incubation for 2 h at 4 °C with the appropriate antibody, which had been pre-coupled to protein G-Sepharose at 4 °C overnight. The immune complexes were washed three times in lysis buffer, separated by SDS-PAGE, and then analyzed by Western blotting. GST pull-down assays were performed using the recombinant GST fusion proteins coupled to GSH-Sepharose. Lysis extracts were prepared as described above and incubated for 2 h with the fusions indicated in the figure legends. The complexes were washed three times in lysis buffer and subjected to SDS-PAGE and Western blotting. Monoclonal antibodies against the epitope tags FLAG (12CA5) and Myc (9E10) were obtained from Sigma. The polyclonal antibody against the HA epitope tag (Y-11) was obtained from Santa Cruz Biotechnology. The monoclonal AU1 antibody was obtained from Berkeley Antibody Company. The polyclonal antibody against human TLR4 was a kind gift from Keith Ray (Glaxo Wellcome, Stevenage, United Kingdom).

RESULTS

Molecular Modeling of TLR4, Mal, and MyD88 TIR Domains—The evidence suggests that TIR domains from receptors and adaptors are likely to form multitypic complexes. To study how this might occur, we used the crystal structures of the TIR domain from TLRs 1 and 2 (17) to generate molecular models for TLR4, Mal, and MyD88 TIR domains. Structure-based alignments of experimental structures to target sequences generated by FUGUE and COMPARE were used by MODELLER6a for comparative modeling (data not shown). The loop regions were refined using RAPPER (25, 26). Model evaluation using Procheck and PROSAIL revealed that the domains were modeled with no structural violations.

Electrostatic surface representations of the experimental structure of TLRs 1 and 2 and the models of TLR4, Mal, and MyD88 are shown in Fig. 1 oriented to show the "S" face. This surface has a number of conserved solvent-exposed residues including the critical Pro residue in the BB-loop. Interestingly, apart from this area the electrostatic surface potentials are quite distinct, suggesting the possibility of electrostatic complementarity. The regions on the upper left and lower right of the BB-loop are the most interesting. For example, in TLR4, the upper left is positively charged (Arg-705), whereas the corresponding region in Mal is negatively charged (Asp-154). Another characteristic of Mal that differentiates it from TLR4 and MyD88 is the lack of conservation in "box 3," a sequence at the C-terminal of the TIR (see Ref. 12). This region is located at the

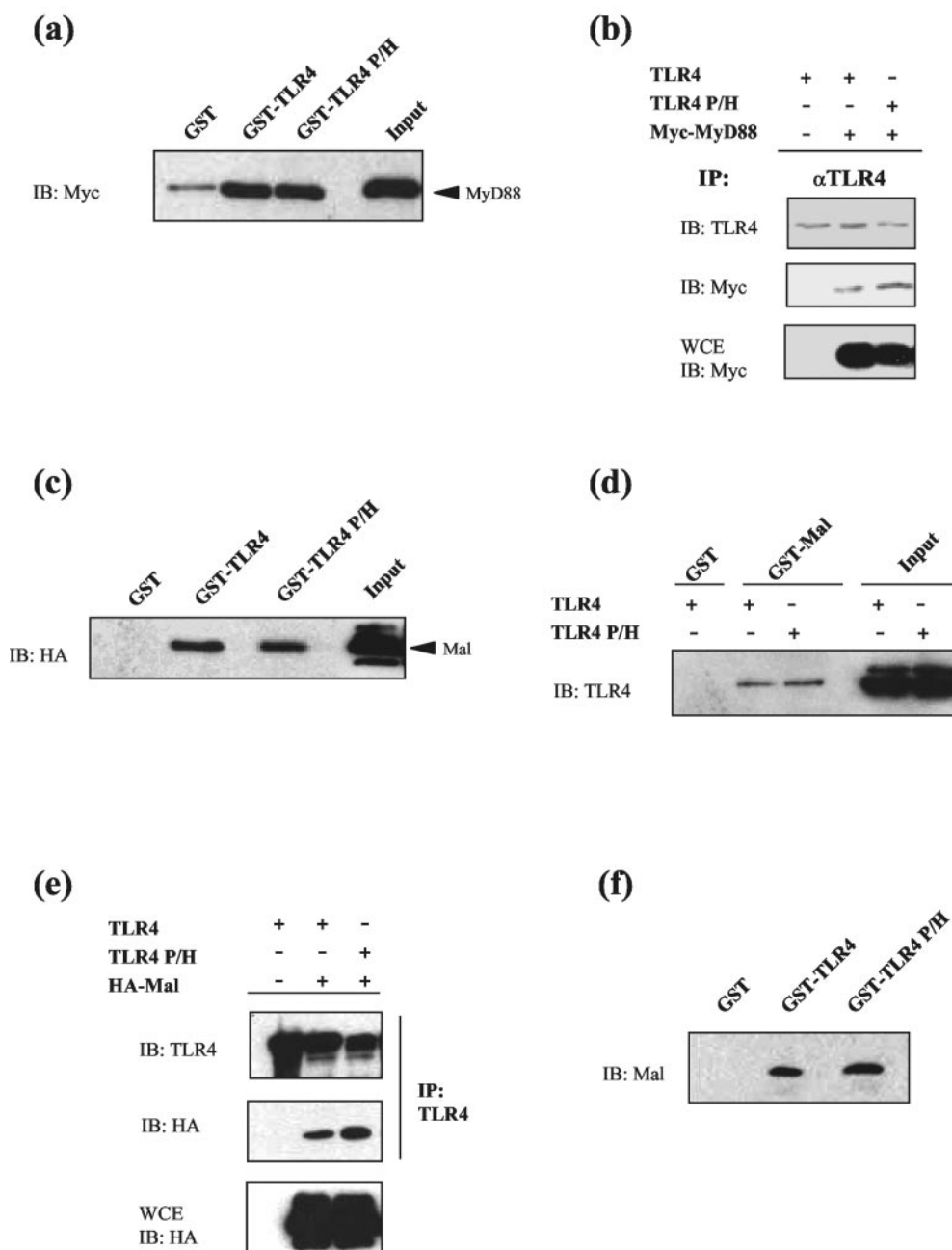


FIG. 3. Wild type and P/H mutant forms of hTLR4 bind Mal and MyD88. HEK293 cells (1×10^6) were transfected with 4 μ g of Myc-MyD88 (a and b), HA-Mal (c and e), TLR4 or TLR4PH (b, d, and e) as shown. Lysates were immunoprecipitated with a polyclonal antibody to human (h) TLR4 or pulled down with the indicated GST construct. Whole cell extracts (WCE) or inputs are shown to demonstrate expression levels. f, purified recombinant Mal was incubated with purified GST-TLR4 (TIR domain) in NETN buffer containing 20 mM Tris, 100 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40. After washing, the complex was analyzed by SDS-PAGE and Western blotting. IB, immunoblotting.

bottom right of the models and in Mal results in a distinct electrostatic surface (Fig. 1e).

As noted above, TLR4, Mal, and MyD88 are able to make heterotypic interactions with each other and these surface properties may indicate how binding specificity is achieved in the post-receptor complex. In an attempt to map the interaction surface for TLR4 with Mal and MyD88, we docked the adaptor proteins onto the TLR4 model using GRAMM (27–29). GRAMM docking was conducted on the basis of hydrophobicity and geometry. Models of the best scoring heterotypic complexes of TLR4 with Mal and MyD88 are presented in Fig. 2a. Interestingly, this study suggests non-overlapping binding sites on TLR4 for Mal and MyD88. Mal is predicted to associate through its DD- and DE-loops with the positively charged re-

gion of TLR4 centered on Arg-705. This region on TLR4 contains one side of the BB-loop and the α C helix and corresponds to the upper left-hand region in Fig. 1d. MyD88 is predicted to bind with a surface created by its AA- and DD-loop to the CD-loop on TLR4, which is located on the other side of the receptor, opposite its BB-loop.

Docking studies with the experimental structure of TLR2 suggest that both adaptors couple to the same surface on this receptor (Fig. 2b). In this case, MyD88 coupling is distinct from that seen with TLR4 with the BB-loop of TLR2 forming a possible point of contact between the two proteins.

Effect of the Mutation Conferring Dominant LPS Sensitivity on Receptor/Adaptor Interactions—Previous modeling studies are comparable with those described above and also represent

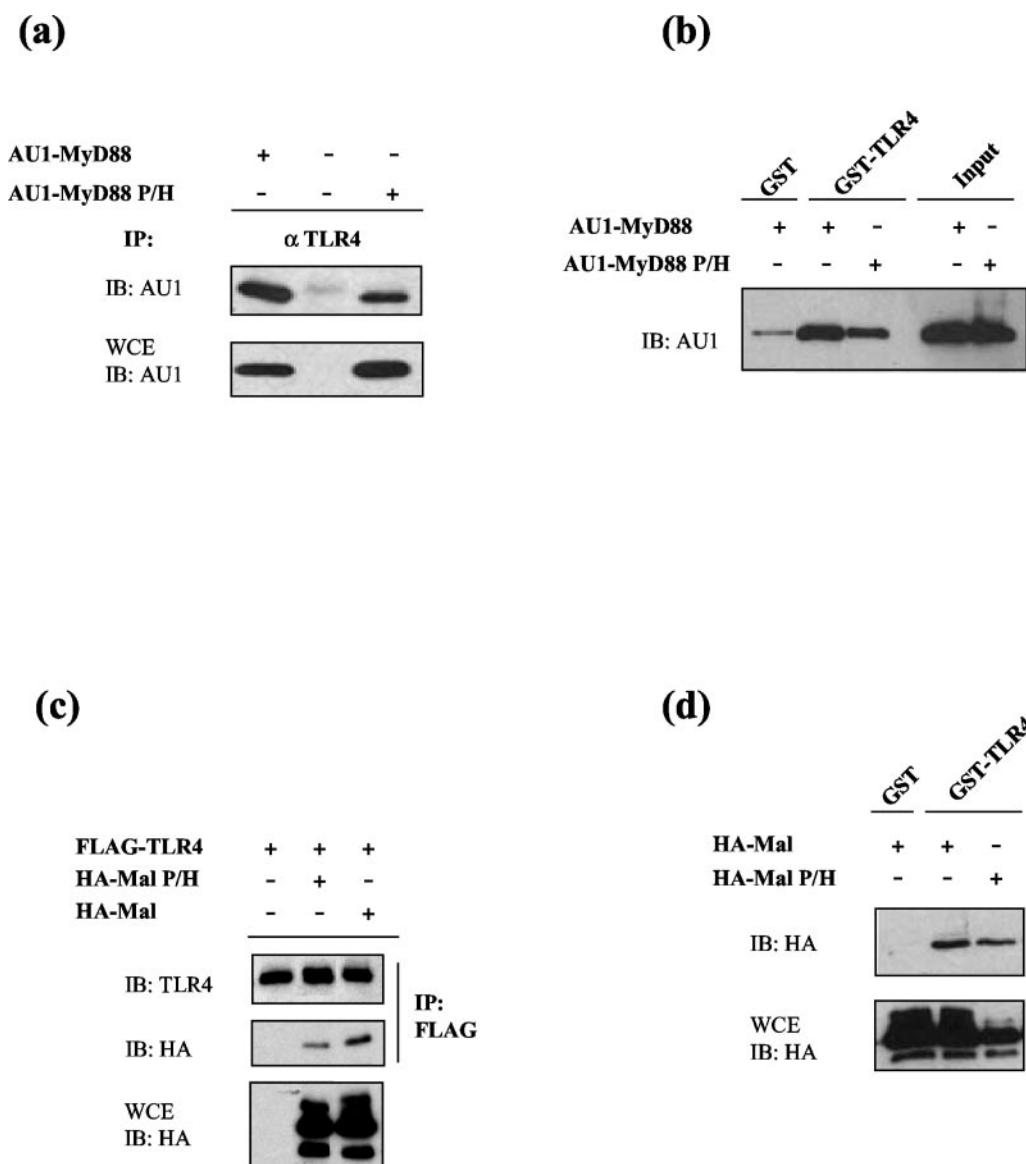


FIG. 4. **Wild type and P/H mutant forms of Mal and MyD88 bind to TLR4.** *a*, HEK293 cells (1×10^6) stably expressing TLR4 were transfected with 4 μ g of AU1-MyD88 or AU1-MyD88 P/H. Lysates were immunoprecipitated (IP) with a polyclonal antibody to TLR4. *b*, lysates from HEK293 cells transfected with the MyD88 constructs were incubated with GST-tagged TLR4. *c*, HEK293 cells were co-transfected with FLAG-tagged TLR4 and HA-Mal or HA-Mal P/H. Lysates were immunoprecipitated with a monoclonal antibody to the FLAG epitope. *d*, HEK293 cells transfected with wild-type and mutant Mal were incubated with GST-TLR4. The resulting complexes were analyzed by SDS-PAGE and Western blotting. IB, immunoblotted; WCE, whole cell extract.

the BB-loop of TLR4 as an exposed surface area (30). Residues within this loop are critical for TLR4 signaling as displayed by the C3H/HeJ mice, which are unresponsive to LPS because of an inactivating proline to histidine mutation at position 712. Mutation of the corresponding residue in human TLR4 (P714H) also renders the receptor inactive and is thought to disrupt MyD88/TLR4 complex formation. This is based on interaction studies with human TLR2 in which the adaptor failed to immunoprecipitate with the mutant receptor (17). According to our model, the P/H mutation should not affect MyD88/TLR4 heterodimerization. Therefore, we carried out similar experiments to clarify whether this is in fact the case. As shown in Fig. 3, *a* and *b*, the TLR4 P/H mutant retained the ability to bind to MyD88 in both co-immunoprecipitation and GST pull-down assays. Both the wild type and mutant forms of TLR4 were also found to bind to Mal in reciprocal GST pull-down assays and co-immunoprecipitations (Fig. 3, *c–e*). This confirms findings from previously reported immunoprecipitations experiments (12, 13). Furthermore, a direct interaction was observed

between wild type and mutant TLR4 and Mal when the purified recombinant proteins were incubated in isolation (Fig. 3*f*).

A corresponding proline residue is also found in the BB-loops of Mal and MyD88. Therefore, we tested the ability of mutant forms of the adaptors to bind to TLR4. As shown in Fig. 4, *a* and *b*, the MyD88 P200H mutation has no effect on TLR4/MyD88 heterodimerization and is consistent with our model in which the BB-loop of MyD88 does not form a point of contact with the receptor TIR domain. It was previously reported that the Mal P125H mutant (which fails to activate NF κ B) cannot bind to TLR4 (13); however, in our hands, an interaction was observed between the two proteins in co-immunoprecipitation and GST pull-down experiment (Fig. 4*c*). Again, this is consistent with our model in which the BB-loop of Mal is directed away from the receptor.

Mal and MyD88 Heterodimerization—GRAMM docking predicts that Mal and MyD88 TIRs interact with each other at a third non-overlapping site (Fig. 5*a*). This interface produces an essentially symmetrical arrangement of the Mal and MyD88

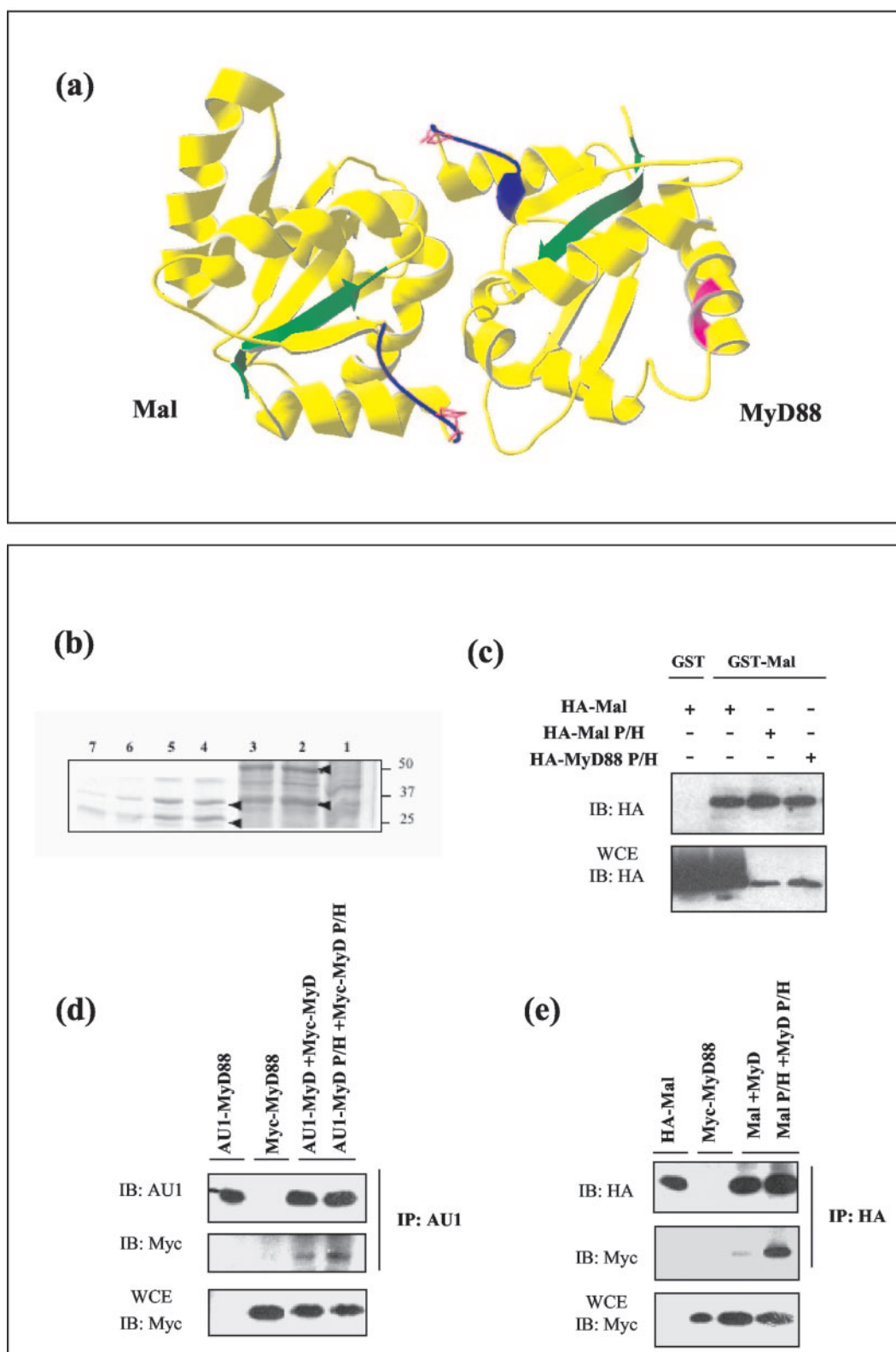


FIG. 5. Homodimerization and heterodimerization of the adaptors is not influenced by the Pro/His mutation. *a*, results of GRAMM docking of Mal (left) and MyD88 (right) TIR domains. Structural features representing the conserved boxes of the TIR domains are shown in green (box 1), blue (box 2, BB-loop), and purple (box 3, not present in Mal). The side chain of the semi-conserved proline residue in the BB-loop is colored purple. *b*, association of Mal with MyD88. GST-Mal (upper arrow) and His-Myd88 (lower arrow) were co-expressed in *E. coli* BL21(DE3) cells (lane 1 (uninduced) and lanes 2–3 (induced with isopropyl-1-thio- β -D-galactopyranoside)). Cleaved GST-Mal (lower arrow) and His-MyD88 (upper arrow) co-elute after thrombin cleavage on a glutathione-Sepharose resin (lanes 4–7). In *c–e*, HEK293 cells (1×10^6) were transfected with 4 μ g of the isolated wild type and mutant TIR domains of Mal and/or MyD88 as indicated. *c*, homodimerization and heterodimerization of full-length Mal was studied in a GST pull-down experiment with GST-Mal and HA-tagged Mal, Mal P/H or Myd88 P/H. *d*, MyD88 TIR domain homodimerization was studied using AU1- and Myc-tagged TIR-domain constructs of the wild type and P/H form of MyD88. Lysates were immunoprecipitated with a monoclonal antibody to the AU1 epitope. *e*, heterodimerization of the TIR domains of Mal and MyD88 and their P/H forms was studied using HA-tagged Mal and Myc-tagged MyD88. Lysates were immunoprecipitated with a polyclonal antibody to the HA tag.

protomers with hydrophobic residues at the end of the BB-loop (Ile-30 and Val-205, respectively) together with polar residues on the fourth α helix (Gln-153, Asp-154 in Mal, and Gln-230 in MyD88 in Fig. 5a) making significant contributions. To address whether this interaction is direct, we co-expressed a His-tagged version of full-length Myd88 and GST-tagged full-length Mal in *E. coli* BL21(DE3) cells (Fig. 5b, lanes 1–3). The two proteins were purified by affinity chromatography on a glutathione-Sepharose column. After extensive washing, the bound proteins were eluted by thrombin cleavage. Myd88 was observed to co-elute with Mal (Fig. 5b, lanes 4–7).

Mutation of Pro-125 in Mal had no effect on the association of Mal with itself or with MyD88 in pull-down and co-immunoprecipitation experiments (Fig. 5, c and e). Likewise, mutation of the corresponding proline residue in MyD88 (P200H) did not prevent its homodimerization as shown in Fig. 5d. Importantly, a construct consisting only of the TIR domain of MyD88 was used in these experiments to rule out any N-terminal interactions that may be mediated by the death domain of MyD88.

ATP-dependent Dissociation of Mal Dimers and Oligomers—When studying some of the homotypic and heterotypic interactions described above, the TIR domains of Mal and MyD88 were expressed in *E. coli* (see “Experimental procedures”). As a final step of purification, the Mal and MyD88 preparations were fractionated by gel-filtration chromatography on Superdex 75 16/60. This analysis revealed an unexpected adenine nucleotide-dependent behavior by Mal. Soluble fractions of induced cells were prepared in either the presence or absence of 1 mM ATP, a treatment reported to remove a contaminant that otherwise co-purifies with GST fusion proteins. The extracts were then purified as described above and finally separated by gel filtration. As shown in Fig. 6, untreated Mal elutes in three well resolved peaks, all of which consist of pure Mal protein. By contrast, Mal purified from ATP-treated lysates migrates as a single peak. This result was reproduced in several experiments and was not observed with corresponding MyD88 TIR domain preparations (data not shown).

Sequence Relationship between Mal and the *Drosophila* Adaptor Tube—The adaptors dMyD88 and Tube are known to couple to the *Drosophila* receptor, Toll. Because dMyD88 is a direct homolog of vertebrate MyD88, we asked whether Mal might be related to Tube. We generated an alignment between the two proteins using ClustalW and found that the N-terminal sequence of Mal immediately preceding the TIR domain is strikingly similar to the C-terminal of Tube adjacent to the death domain (Fig. 7). 30 of 80 amino acids in this region are conserved or conservatively substituted. These amino acids are also highly conserved in the Tube sequence from the distantly related dipteran *Drosophila virilis* (31). The C-terminal of Tube is also aligned by ClustalW with 47 identities or conservative substitutions in the last 150 amino acids. The most conserved regions correspond to the predicted β -strands in the TIR models and particularly hydrophobic core residues. Thus, it seems possible that the Tube C terminus might represent a highly degenerated member of the TIR-fold family, consistent with its role in coupling activation and signaling by the *Drosophila* Toll receptor.

DISCUSSION

The modeling and docking studies presented here suggest that there are discrete sites on the receptor TIR domains for binding Mal and MyD88 and a third mode of binding for the formation of Mal/MyD88 heterodimers. Neither of the predicted interactions of the adaptor TIR domains appears to directly involve the BB-loop region on TLR4, although that predicted for Mal is immediately adjacent to it. *In vitro* assays confirm that mutation of a critical proline residue, which lies at

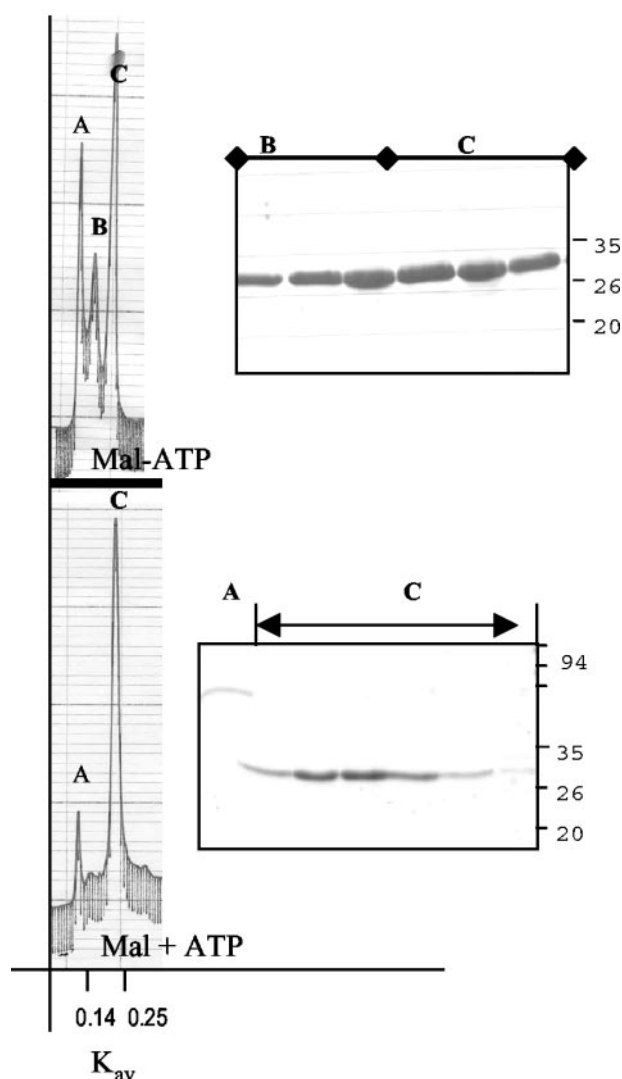


FIG. 6. **ATP-dependent behavior of Mal.** Mal was purified in the absence and presence of ATP and $MgCl_2$ and analyzed by size-exclusion chromatography. Fractions corresponding to the peaks labeled on the elution profile were analyzed by SDS-PAGE.

the tip of the BB-loop is not sufficient to prevent recruitment of the adaptor proteins. This result contrasts with the finding that mutation of the corresponding residue in TLR2 abolishes the binding of MyD88. Importantly, there are no significant structural differences between the wild type and mutant TLR2 protein (17), and this is probably the case with TLR4. Of course, we cannot rule out the possibility that MyD88 is recruited to the mutant receptor via endogenous Mal should the latter bind at a distinct surface. It may also be the case that mutation of a single residue within the BB-loop is not sufficient to prevent receptor/adaptor association. Indeed, a recent report providing a detailed examination of interaction surfaces on the TLR4 TIR domain revealed that amino acids in both the BB- and DD-loops on TLR4 are critical for signaling (30). In addition, the program GRAMM uses a rigid docking procedure and would not be able to predict sites that undergo a significant conformational change on binding. Thus, if the BB-loop is directly involved in binding, it may undergo a switch such as that seen upon the binding of vonWillebrand factor to platelet glycoprotein 1B (Gp1B) (32). In this case, a 16 amino acid loop in Gp1B becomes a β -hairpin and this forms part of a three-stranded intermolecular antiparallel β -sheet. In support of this finding, a recent study of a mutant TLR2 TIR domain, which assumes

Mal	1	-----
Tube	1	IPKGM DVQACSGAGCLNFP AEIKKGF KYTAQDVFQIDEAANRLPPDQSKSQMMIDEWKTS
consensus	1	
Mal	1	-----
Tube	61	GKLNERTVGVLLQLLVQAE LFSAADFVALDFLN ESTPARPVDGPGALISLELLEEEEME V
consensus	61	
Mal	1	-----MASSTSLPAPGSRPKPLGKMADWFRQTLLKKPKRPN SPESSTSSDASQPTSQD
Tube	121	DNEGLS LKYQSSTATLGADAQGSVGLNLDNFEKDIVRRDKSVPPQPSGNTPTPIAPPRRQQR
consensus	121	* * * * * * * * *
Mal	55	NLP PPSLSSVTSPSLPPTHASDSGSSRWSKDYDVCVCHSEEDLVAAQDLVSYLEGSTASL
Tube	181	STTNSNFATLTGTGTTSTTIPNVPNLITLNPSEQIQEPVLQPRPMNIPDLSILISNSGDL
consensus	181	..* * . * * * *
Mal	115	RCFLQLRDATPGGAIVSEL CQALSSSHCRVLLITPGFLQDPWCKYQMLQALTEAPGAEGC
Tube	241	RATVSDNPSNR-----TSSTDPPNIPRITLLIDNSGDVNSRPNHAPAKASTATTPTASSN
consensus	241	* * * . * *
Mal	175	TIPLLSGLSR-----AAYPPELR-----FMYYVDGRGPDG---GFRQVKEAVMRCKLLQEG
Tube	296	NLPMISALNISKGSKETLRPESSSSSSLSKDDDDNDGEEDGEE EYPDAFLPNLSNSEQ
consensus	301	.*.*.* * * * * * * * * *
Mal	223	EGERDSATVSDLL-----
Tube	356	QSSNNDSSLTTVTGTSGDNSFELTNDSSSTSNDDYACNIPDLSELQQ
consensus	361	

FIG. 7. Alignment of full-length Mal to *Drosophila* Tube. The N-terminal region of Mal shows some striking similarity to a short region C-terminal to the death domain of Tube. Identities are indicated by stars, and conservative substitutions are indicated by dots.

a somewhat different conformation to that in the original crystal structure, indicates that the BB-loop is intrinsically flexible (33).

The BB-loops of the adaptors appear also not to form a point of contact with either TLR4 or themselves in both docking and experimental studies. Again, we cannot rule out the possibility that the mutant proteins are recruited to the receptor complex by their endogenous wild type counterparts. Direct measurement of protein-protein interactions is required to determine the validity of these models.

We have expressed and purified Mal and MyD88 to study them biochemically. We have shown that heterodimers of MyD88 and Mal can be reconstituted in a purified system consistent with previous results using immunoprecipitation and yeast two-hybrid assays (12). We have also shown that the Mal protein can exist in different oligomeric states including dimers. This is also consistent with yeast two-hybrid experiments, which indicated the ability of Mal to form homodimers as well as heterodimers with MyD88. Unexpectedly, we observed that the Mal oligomers could be dissociated if they were prepared in the presence of high concentrations of ATP. The significance of this behavior is unclear; however, it suggests

that Mal can adopt different conformers and that ATP can directly or indirectly convert these forms. Direct purine nucleotide binding has been proposed previously for TIR domains (34). In addition, the TIR-fold is very similar to that of CheY, a bacterial chemotaxis protein that is transiently phosphorylated at an aspartate residue. This phosphorylation induces subtle changes in the CheY structure, particularly the change in conformation of a tyrosine residue in the loop between the fourth β -sheet and fourth α -helix. This conformational switch modulates the interaction of CheY with the flagellar motor, membrane sensor CheA, and regulator CheZ (35). Given the remarkable structural conservation between CheY and the TIRs, it seems possible that the function of these homologous signaling domains also involves conformational changes induced by nucleotide binding or phosphotransfer.

Finally, our analysis suggests that the Mal protein is related to Tube, an adaptor for the *Drosophila* Toll receptor. Until recently, it was thought that Tube was sufficient to establish signaling in the *Drosophila* Toll pathway. However, it is now clear that a direct homologue of vertebrate MyD88 is required in addition to Tube for both dorso-ventral patterning and for antifungal responses (15, 36), a situation reminiscent of the

functional interdependence between vertebrate Mal and MyD88. Tube has a death domain sequence at the N-terminal, and this alone can restore some level of signaling by the Toll receptor (31). The homology identified is principally located at the N-terminal of Mal preceding the TIR, a region with a number of potential phosphorylation sites, which could fulfill a regulatory role. In contrast to Tube, Mal does not have a death domain and must therefore couple to the downstream signaling pathway in a manner distinct from MyD88. The functional interrelationships of Mal, Tube, and *Drosophila* and vertebrate MyD88 homologues suggest that the C-terminal of Tube serves a role comparable with that of a TIR and may be a highly degenerated example of this fold, which cannot be detected from primary sequence identity.

These studies point to a mechanism by which TIR adaptors can be recruited in response to activation of the receptor. A further understanding of this problem requires the experimental determination of structures for receptor and adaptor TIR complexes. The emergence of additional TLR signaling adaptors adds further complexity to this question, and it is clear that extensive *in vitro* studies are required to understand the precise mechanism by which TLRs assemble into higher order complexes.

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REFERENCES

- Dunne, A., and O'Neill, L. A. (2003) *Science's STKE* <http://stke.sciencemag.org/cgi/content/full/sigtrans;2003/171/re3>.
- Gay, N. J., and Keith, F. J. (1991) *Nature* **351**, 355–356.
- Belvin, M. P., and Anderson, K. V. (1996) *Annu. Rev. Cell. Dev. Bi.* **12**, 393–416.
- Imler, J. L., and Hoffmann, J. A. (2001) *Trends Cell Biol.* **11**, 304–311.
- Buchanan, S. G. S., and Gay, N. J. (1996) *Prog. Biophys. Mol. Biol.* **65**, 1–44.
- Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A., and Bazan, J. F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 588–593.
- Triantafyllou, M., and Triantafyllou, K. (2002) *Trends Immunol.* **23**, 301–304.
- Poltorak, A., He, X. L., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) *Science* **282**, 2085–2088.
- Wesche, H., Henzel, W. J., Shillinglaw, W., Li, S., and Cao, Z. D. (1997) *Immunity* **7**, 837–847.
- Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) *Immunity* **11**, 115–122.
- Hardiman, G., Rock, F. L., Balasubramanian, S., Kastelein, R. A., and Bazan, J. F. (1996) *Oncogene* **13**, 2467–2475.
- Fitzgerald, K. A., Palsson-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Mansell, A. S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. T., McMurray, D., Smith, D. E., Sims, J. E., Bird, T. A., and O'Neill, L. A. J. (2001) *Nature* **413**, 78–83.
- Hornig, T., Barton, G. M., and Medzhitov, R. (2001) *Nat. Immunol.* **2**, 835–841.
- Hornig, T., and Medzhitov, R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 12654–12658.
- Tauszig-Delamasure, S., Bilak, H., Capovilla, M., Hoffmann, J. A., and Imler, J. L. (2002) *Nat. Immunol.* **3**, 91–97.
- Letsou, A., Alexander, S., Orth, K., and Wasserman, S. A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 810–814.
- Xu, Y. W., Tao, X., Shen, B. H., Hornig, T., Medzhitov, R., Manley, J. L., and Tong, L. (2000) *Nature* **408**, 111–115.
- Simonovic, M., and Volz, K. (2001) *J. Biol. Chem.* **276**, 28637–28640.
- Sali, A., and Blundell, T. L. (1990) *J. Mol. Biol.* **212**, 403–428.
- Shi, J., Blundell, T. L., and Mizuguchi, K. (2001) *J. Mol. Biol.* **310**, 243–257.
- Mizuguchi, K., Deane, C. M., Blundell, T. L., Johnson, M. S., and Overington, J. P. (1998) *Bioinformatics* **14**, 617–623.
- Burke, D. F., Deane, C. M., Nagarajaram, H. A., Campillo, N., Martin-Martinez, M., Mendes, J., Molina, F., Perry, J., Reddy, B. V. B., Soares, C. M., Steward, R. E., Williams, M., Carrondo, M. A., Blundell, T. L., and Mizuguchi, K. (1999) *Proteins*, 55–60.
- Laskowski, R. A., Macarthur, M. W., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Crystallogr.* **26**, 283–291.
- Morris, A. L., Macarthur, M. W., Hutchinson, E. G., and Thornton, J. M. (1992) *Proteins* **12**, 345–364.
- de Bakker, P., DePristo, M., Burke, D., and Blundell, T. (2003) *Proteins* **51**, 21–40.
- DePristo, M., de Bakker, P., Lovell, S., and Blundell, T. (2003) *Proteins* **51**, 41–55.
- Katchalski-Katzir, E., Shariv, I., Eisenstein, M., Friesem, A. A., Aflalo, C., and Vakser, I. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 2195–2199.
- Vakser, I. A. (1995) *Protein Eng.* **8**, 371–377.
- Wells, C. A., and Vakser, I. A. (2002) *Protein Sci.* **11**, 1888–1896.
- Ronni, T., Agarwal, V., Haykinson, M., Haberland, M. E., Cheng, G., and Smale, S. T. (2003) *Mol. Cell. Biol.* **23**, 2543–2555.
- Letsou, A., Alexander, S., and Wasserman, S. A. (1993) *EMBO J.* **12**, 3449–3458.
- Huizinga, E. G., Tsuji, S., Romijn, R. A. P., Schiphorst, M. E., de Groot, P. G., Sixma, J. J., and Gros, P. (2002) *Science* **297**, 1176–1179.
- Tao, X., Xu, Y. W., Zheng, Y., Beg, A. A., and Tong, L. (2002) *Biochem. Biophys. Res. Commun.* **299**, 216–221.
- Hopp, T. P. (1995) *Protein Sci.* **4**, 1851–1859.
- Zhu, X. Y., Volz, K., and Matsumura, P. (1997) *J. Biol. Chem.* **272**, 23758–23764.
- Kambris, Z., Bilak, H., D'Alessandro, R., Belvin, M., Imler, J. L., and Capovilla, M. (2003) *EMBO Rep.* **4**, 64–69.