Interactive Sites in the MyD88 Toll/Interleukin (IL) 1 Receptor Domain Responsible for Coupling to the IL1β Signaling Pathway*

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Myeloid differentiation factor MyD88 is the essential adaptor protein that integrates and transduces intracellular signals generated by multiple Toll-like receptors including receptor complex for interleukin (IL) 1β, a key inflammatory cytokine. IL1β receptor complex interacts with MyD88 via the Toll/IL1 receptor (TIR) domain. Here we report structure-function studies that help define the MyD88 TIR domain binding sites involved in IL1β-induced protein-protein interactions. The MyD88 TIR domain, employed as a dominant negative inhibitor of IL1β signaling to screen MyD88 TIR mutants, lost its suppressing activity upon truncation of its Box 3. Accordingly, mutations of Box 3 residues 285–286 reversed the dominant negative effect of the MyD88 TIR domain on IL1β-induced and NFκB-dependent reporter gene activity and IL6 production. Moreover, mutations of residues 171 in helix αa, 195–197 in Box 2, and 275 in βE-strand had similar functional effects. Strikingly, only mutations of residues 195–197 eliminated the TIR-TIR interaction of MyD88 and IL1 receptor accessory protein (IL1RAcP), whereas substitution of neighboring canonical Pro200 by His was without effect. Mutations in Box 2 and 3 prevented homotypic MyD88 oligomerization via TIR domain. Based on this structure-function analysis, a three-dimensional docking model of TIR-TIR interaction between MyD88 and IL1RAcP was developed.

The importance of the Toll-like receptor (TLR) family in innate immune response to microbial surfaces and nucleic acids is well established. Surprisingly, two members of this family recognize key inflammatory cytokines interleukin (IL) 1 and IL18. These cytokines induce genes that encode other mediators of inflammation such as pleiotropic inflammatory cytokine IL6 and interferon γ, respectively (1–5).

Consistent with these studies, IL1β is one of the most potent inflammatory cytokines responsible for fever, leukocytosis, thrombocytosis, and production of IL6 and other cytokines (1–3). The signals generated by IL1β binding to its cognate receptor complex, formed by two type I transmembrane proteins, IL1 receptor I (IL1RI) and IL1 receptor accessory protein (IL1RAcP), are transduced by their cytoplasmic segments denoted as the Toll/IL1 receptor (TIR) domain. TIR domain is shared with Drosophila Toll, mammalian TLRs, and cytoplasmic adaptors exemplified by MyD88 (6).

MyD88 adaptor integrates signals flowing from IL1 receptor/IL1RAcP and from an array of other TLRs (6, 7). This initial IL1 receptor-MyD88 adaptor interaction evoked by IL1β is a critical step in its signaling to the nucleus and, therefore, represents a potential target for new anti-inflammatory agents. MyD88 has a bipartite structure composed of an aminoterminal Death domain and a carboxyl-terminal TIR domain with a short intervening linker segment (6). Upon IL1β stimulation, IL1RI/IL1RAcP complex recruits MyD88 via its TIR domain (8). In addition, IL1RI-associated kinases are recruited to an IL1RI/IL1RAcP complex including IRAK (9, 10), IRAK-2 (11), IRAK-4 (12) and IRAK-M (13). Our current understanding of IL1 receptor complex-MyD88 adaptor interaction is limited. Here we report studies that help to establish the molecular determinants of MyD88 TIR domain interactions in IL1β signaling pathway.

In terms of its structural features, the MyD88 TIR domain contains three highly conserved motifs denoted Box 1, 2, and 3 (Figs. 1 and 2). Box 2 forms a loop denoted the BB loop that contains an invariant proline residue at position 200, which, in other receptors and adaptors (namely, TLR2, TLR4, IL1RAcP, and MAL/TIRAP), is essential for their signaling function (6, 7). For example, mutating this residue to histidine in TLR4 renders C3H/HeJ mice hyporesponsive to lipopolysaccharide (LPS) (14). This canonical example indicates that conserved structural motifs in TIR domain of TLRs and their adaptors play a highly significant role in proinflammatory ligand-initiated intracellular interactions between TLRs and their adaptors. Depending on the recognition of distinct ligands by TLRs, the preferential usage of its adaptors may require different interacting sites in TIR domain of the same adaptor or an alternative adaptor. The latter applies to TLR3, which requires its adaptor, TRIF, rather than MyD88 for signaling by viral double-stranded RNA. Conversely, TRIF mediates signaling induced by interaction of LPS with TLR4 in the absence of MyD88 (7). We hypothesized that signaling evoked by IL1β through its cognate receptor complex may depend on different interactive sites on TIR domain of MyD88 than the recently reported sites involved in MyD88 interaction with TIR domains of TLR2 and TLR4 (15).

To test this hypothesis, we undertook our studies focused on the potential role of Box 1, 2, and 3 of MyD88 TIR domain in
IL1β signaling. Within these three boxes, we focused on residues representing AA loop, BB loop, and EE loop that were reported to participate in interactions of MyD88 with TLR2 and TLR4 (15). The functional consequences of this mutational analysis were monitored by NFκB-dependent reporter gene activity and by IL1β-induced expression of the endogenous gene that encodes inflammatory cytokine IL6. Our structure-function studies of MyD88 TIR domain led to the development of a three-dimensional docking model of MyD88 and IL1RαcP interaction mediated by their respective TIR domains.

EXPERIMENTAL PROCEDURES

Maintenance and Treatment of Cell Lines—Human embryonic kidney (HEK) 293T cells and human fibroblast MRC-5 cells were obtained from the American Type Culture Collection (Manassas, VA). HEK 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% heat-inactivated fetal bovine serum containing no detectable LPS (<6 pg/ml) as determined by the manufacturer (Atlanta Biological, Norcross, GA), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). MRC-5 cells were maintained in minimum Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). All cells were maintained at 37°C in a humidified atmosphere of 5% CO2.

Plasmids and Reagents—The NFκB-luciferase reporter construct (NFκB-luc) containing five kB elements was provided by Dean Ballard (Vanderbilt University, Nashville, TN). The Renilla thymidine kinase luciferase reporter construct (RL-TK luc) was purchased from Promega. The AU1-tagged MyD88-expressing plasmid was a gift from Marta Muzio (Mario Negri Institute, Milan, Italy) (16). All MyD88-TIR constructs were cloned into pcDNA3.1. An AU1 tag or a Myc tag was introduced at the amino terminus of MyD88 or MyD88-TIR by PCR. IL1RαcP with a Myc tag at the amino terminus was cloned by reverse transcription-PCR into pcDNA3.1. All constructs were verified by sequencing.

Mutagenesis—The mutated MyD88 and MyD88 TIR domain sequences were generated using an in vitro mutagenesis method and subcloned into plasmid pcDNA3.1 (Invitrogen) as described previously (17). Briefly, PCR was utilized with a supercoiled double-stranded DNA template and two synthetic complementary oligonucleotides containing the desired mutation and followed by removal of methylated parental DNA template with DpnI. The nicked DNA containing the desired mutations was transformed into the DH5α strain of competent Escherichia coli (Invitrogen). All the mutants were confirmed by DNA sequencing and subsequently tested in transiently transfected HEK 293T cells.

Transient Transfection of HEK 293T Cells and NFκB Reporter Gene Activity—The cDNAs for all MyD88 and MyD88 TIR domain mutants were inserted into the pcDNA3.1 vector that drives transcription from a cytomegalovirus promoter enhancer region, and templates for immunodetection of transiently expressed mutants in HEK 293T cells. Transfection of HEK 293T cells was performed with the indicated cDNAs by a conventional calcium phosphate method. One day before transfection, cells were seeded at a density of 2.5 × 10⁴ cells/100-mm plate. After 18 h, the culture medium was replaced with fresh medium, and after 24 h, the cells were treated as indicated, harvested, and subjected to subsequent analysis. When the cells were stimulated with LPS, the culture medium was replaced with fresh medium containing LPS (CellSciences, Canton, MA) at 10 ng/ml or as indicated otherwise and either further cultured for 6 h or left untreated for the indicated period of time.

Western Blotting and Indirect Immunofluorescence—To check the protein expression after transient transfection, HEK 293T cells were seeded (2.5 × 10⁵ ml⁻¹) onto 100-mm dishes 24 h prior to transfection with combinations of plasmids (20 μg, total) or as indicated, using calcium phosphate method. Thirty-six hours after transfection, cells were washed by the addition of 5 ml of ice-cold phosphate-buffered saline. Cells were lysed on ice for 10 min in lysis buffer containing 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM Na3VO4, and 1 μM ml⁻¹ leupeptin. Cell lysate proteins (50 μg) were separated by SDS-PAGE and then analyzed by Western blotting. Monoclonal antibodies against the epitope tags e-Myc and AU1 were obtained from Covance Company (Princeton, NJ). For indirect immunofluorescence, 10⁶ transfected HEK 293T cells were cytocoentrifuged onto a glass slide and fixed with 3.5% paraformaldehyde. After washing with phosphate-buffered saline, cells were permeabilized with 0.25% Triton X-100 for 10 min and then blocked with 10% anti-AU1 antibody followed by Rhodamine Red X-labeled goat anti-mouse IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) as described previously (18). Slides with stained cells were mounted in Poly(Mount) (Polysciences, Warrington, PA) and analyzed in an Olympus fluorescence microscope using a 100 × oil immersion lens.

Measurement of IL6 Expression Using Cytometric Bead Array Assay—One million MRC-5 cells were transfected with Cell Line Nucleofector Kit R (Amuax, Gaithersburg, MD) program U23, following the manufacturer’s protocol. After 8 h, cells were washed with Hank’s balanced salt solution, and fresh media were added. Cells were stimulated with either IL1β for 6 h, and culture medium was collected and analyzed for the production of cytokine IL6. Analysis of IL1β-induced expression of cytokine IL6 in human fibroblast MRC-5 cells was performed using the Human Inflammation Kit (BD Biosciences) according to the manufacturer’s protocol.

Immunoprecipitation—HEK 293T cells were plated at the density of 5 × 10⁵ cells/100-mm plate. Twenty-four hours later, cells were transfected with either (a) 10 μg of Myc-IL1RαcP and 10 μg of AU1-Myc-MyD88 or MyD88 mutants or (b) 10 μg of Myc-MyD88 and 10 μg of AU1-TIR or TIR mutants using calcium phosphate method. Fresh medium was added 18 h later, and cells were incubated for 24 h. In case of co-transfection of IL1RαcP and MyD88, the cells were treated with 100 ng/ml IL1β for 5 min. The cells were washed with phosphate-buffered saline and resuspended in 400 μl of hypotonic gentle lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, and 1 μM leupeptin). Lysates were initially precleared with normal mouse serum in combination with protein A-Sepharose 4 fast flow beads (Amersham Biosciences), and then 200 μg of lysate was incubated with 10 μg of anti-Myc at 4°C overnight. Protein A-Sepharose 4 fast flow slurry (10 μl) was added and incubated for 3 h at 4°C. Beads were washed eight times with 0.5 ml of wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, and 1 μM leupeptin). Beads were resuspended in 20 μl of 2× Laemmli buffer. The samples were transferred to 15% SDS-PAGE and run. Hydroxylapatite (MRC-5), the culture medium was replaced by fresh medium containing IL1RAcP and TIR Interaction—The best loop was selected using a score scheme including force field energy, steric hindrance, and specific or nonspecific interaction, coordinates. The best loop was selected using a score scheme including force field energy, steric hindrance, and specific or nonspecific interaction, hydrogen bond formation or dipole-dipole interaction. Side chains were reconstructed by weighting positions of corresponding residues in the sequence, were then prepared to create the core of the model by averaging four steps: template selection (BLASTP2), target-template alignment (HSSMOS96). The visualizations of the three-dimensional model were performed with the DeepView (Swiss-PdbViewer) freeware program, available for download from the Swiss-Model webpage.

The three-dimensional models of MyD88 and IL1RαcP TIR domains were established by a comparative (homology) computation modeling method using Swiss-Model: An Automated Comparative Modeling Server (swissmodel.expasy.org). All the HSSMOS96. The visualizations of the three-dimensional model were performed with the DeepView (Swiss-PdbViewer) freeware program, available for download from the Swiss-Model webpage.

The Three-dimensional Docking Model of MD88 TIR and IL1RαcP TIR Domains—Many aspects associated with the model such as monodispersity, surface topology, charge distribution, electrostatic field, and residue localization, were considered before an arrangement was constructed. First, a possible position of two interacting TIR domains was prepared manually in stereoview mode with the PSSHOW program (SYBYL-Tripos package). When the proper position was chosen, computation was conducted by merging receptor (IL1RαcP-TIR) into adaptor (MyD88-TIR), coordinates of the backbone atoms were then frozen, and...
the heavy atom aggregate was created and optimized. In the next step, hydrogen atoms were added, and the final model was optimized by energy minimization followed by molecular dynamics. Both steps were performed with the SANDER program (AMBER software package) using integral Newtonian equation of motion with 2000 cycles each. Once the completion process was completed, the coordinates were transformed into a Protein Data Bank file. The three-dimensional docking models of dot-surface and contact surface were obtained with PSS-HOW software. The ribbon structure of docking model was prepared with DeepView (Swiss-PdbViewer).

**RESULTS AND DISCUSSION**

The IL1β signaling pathway depends on an orchestrated interplay of intracellular protein-protein interactions (1–3). MyD88 plays a pivotal role in these interactions by directing the flow of signals from IL1β-occupied cognate receptor complex to downstream signal transducers (2, 3). Within MyD88, the TIR domain provides an interacting surface for heterotypic interaction with the TIR domain of IL1RAcP (3). Therefore, we embarked on structure-function analysis of MyD88 TIR domain that is essential for the transduction of IL1β signaling to downstream effector(s). Our stepwise strategy consisted of analysis of the secondary and tertiary structure of MyD88 TIR domain. These data were obtained on the basis of the available crystal structure of TLR2 TIR domain (21). Drawing from these results, we solved for TLR2 (21). It has an Aβ-strand similar to that of the bacterial chemotaxis protein CheY and contains three highly conserved motifs, Box 1, located at the amino terminus of TIR domain. Box 2 makes the second part of the BB loop, whereas Box 3 creates the first part of the α-helix, which is located at the carboxyl terminus of MyD88 TIR domain. As shown in Fig. 2B, distribution of charged residues indicates that the molecular surface of MyD88 TIR domain is mostly positively charged (blue), with a few distinct negatively charged knobs (red). They surround a larger swath of negatively charged surface (red), formed by three loops (AA, BB, and part of DD) and α-helix. Moreover, the BB loop projects from the globular TIR domain, forming a quasi-plane on its surface.

**NFκB Reporter Gene Activity Assay Indicates that Box 3 of MyD88 TIR Domain Is Involved in IL1β-induced Signaling—**

NFκB reporter gene activity assay was used to test MyD88 TIR domain as a dominant negative inhibitor of IL1β-induced signaling to the nucleus. As documented in Fig. 3, HEK 293T cells transfected with plasmid containing NFκB-dependent luciferase gene responded to sub-nanogram doses of IL1β. This activation reached the maximum at 1 ng/ml IL1β, attesting to the high sensitivity of transfected HEK 293T cells to this inflammatory cytokine. Consistent with prior studies (23, 24), the MyD88 TIR domain, used as a dominant negative inhibitor of IL1β signaling, almost completely suppressed its activating effect on NFκB reporter gene over a wide range of IL1β concentrations (Fig. 3). We engineered a deletion mutant of MyD88 TIR domain to establish the utility of the NFκB-dependent luciferase gene reporter assay for screening TIR domain mutants for their inhibitory effect on IL1β-induced signaling. The deleted segment encompassed Box 3 (225–282) located in the carboxyl terminus of MyD88 TIR domain. This deletion caused the loss of the dominant negative inhibitory function of MyD88 TIR (Fig. 3B), suggesting that Box 3 was essential for IL1β-induced signaling. The carboxy-terminal deletion mutant was expressed in transfected cells at a level comparable with that of the intact MyD88 TIR domain (see Fig. 3B, inset).

**Mutagenesis of MyD88 TIR Domain: Loss of Its Dominant Negative Inhibitory Activity toward IL1β-induced Signaling—**

The involvement of Box 3, as compared with Boxes 1 and 2, in signaling induced by IL1β and mediated by the MyD88 TIR domain was analyzed in the first series of mutagenesis experiments. Mutations of TIR domain (residues 152–296) included two bulky hydrophobic residues (Phe285 and Trp286) in a highly conserved short motif in Box 3 (SWFWTRL) and proline at position 200. The canonical P712H mutation in TLR4 renders
C3H/HeJ mice hyporesponsive to LPS (14). This highly conserved proline residue is located at position 200 in the MyD88 TIR domain (15). It was mutated to histidine in MyD88-TIR domain (residues 152–296 (P200H)). Furthermore, alignment of members of the TLR family reveals two short motifs in Box 1 (PERFDAF) and Box 2 (DRDVLPG) that are conserved along with Box 3 motif in MyD88 TIR domain. Alanine substitutions were primarily based on selection of charged residues either in the conserved region or predicted to be on the surface. In addition, two mutations, Val204 and Gln229, were in the region predicted to interact with MAL (15). The expression of mutants varied as compared with that of wild-type MyD88 TIR domain (data not shown). Five mutants listed in Table I were expressed at a level comparable with that of wild-type MyD88 TIR domain (see Fig. 4B, inset). The expression of other mutants was

**Table I**

Information about the MyD88 TIR mutants analyzed in this study

All the TIR mutations were generated as indicated under “Experimental Procedures.” MyD88 TIR mutants that did not inhibit NFκB reporter gene activity by >2.5-fold were considered loss of inhibition phenotype as compared with the dominant negative inhibitory effect of wild-type MyD88 TIR domain.

| Mutant | Mutation(s) | Location | Loss of inhibition |
|--------|-------------|----------|--------------------|
| T1     | D171A       | AA loop<sup>a</sup> | Yes                |
| T2     | D195A/R196A/D197A | Box 2  | Yes                |
| T3     | P200H       | BB loop<sup>b</sup> | No                 |
| T4     | D275A       | βE-strand | Yes                |
| T5     | F285A/W286A | Box 3    | Yes                |

<sup>a</sup> Predicted interactive site of TLR4 (see Ref. 15).

<sup>b</sup> Similar to TLR4 P712H mutation in C3H/HeJ mice Lps<sup>e</sup> allele (see Ref. 14).
reduced or undetectable, presumably due to misfolding and/or degradation. The mutants listed in Table I were screened for potential inhibitory effect on NFkB reporter gene activation following stimulation with IL1β.

Of particular significance is the result with the P200H mutant (T3), analogous to the ProHis mutation in TLR4, which is responsible for the LPS hypersensitivity of C3H/HeJ mice (14). Similar loss of signaling in other TIR-containing molecules such as MAL/TIRAP (25) and IL1RaCp (26, 27) has been reported, indicating that the invariant proline in the BB loop of Box 2 in these molecules is one of the interactive sites for other TIR domain-containing proteins. In striking contrast, a similar mutation (P200H) in MyD88 TIR domain, tested within a range of input concentrations, did not change the dominant negative effect of MyD88 TIR on IL1β-induced NFkB reporter gene activation in 293T cells (Fig. 4A). These cells showed a similar level of expression of wild-type and mutant proteins (Fig. 4B, inset). This result is consistent with recent modeling studies of the interaction of MyD88 with TLRs.

In contrast to the canonical P200H mutation, the following mutants displayed >2.5-fold loss of the dominant negative effect on IL1β-stimulated NFkB reporter gene activation as compared with the wild-type MyD88 TIR domain (Fig. 4B): D171A in helix αA (T1), triple mutant D195A/R196A/D197A (T2) in Box 2, D275A in βα-strand (T4), and double mutant F285A/W286A (T5) in Box 3. The result with the F285A/W286A mutant is consistent with the loss of inhibition displayed by Box 3-deleted MyD88 TIR domain (Fig. 3B). All these mutants and the wild-type MyD88 TIR domain were expressed at a comparable level in HEK 293T cells (Fig. 4B, inset).

These mutants were chosen for further validation of the functional significance of mutated MyD88 TIR domain residues. We selected IL1β-induced expression of an endogenous gene that encodes inflammatory cytokine, IL6, in human fibroblast MRC-5 cells for testing MyD88 TIR domain mutants. The expression of the IL6 gene is regulated by NFkB, and its mobilization by IL1β is dependent on MyD88 (1–3). Upon stimulation of MRC-5 cells with IL1β, an inflammatory cytokine, IL6, was expressed. This expression of endogenous IL6 gene was suppressed 2.5-fold by the dominant negative TIR domain of MyD88 (Fig. 4C).

Mutagenesis of Full-length MyD88 Reveals an Interactive Site for Direct Contact with IL1RaCp—It is still unknown which of the interactive sites identified in the MyD88 TIR domain are responsible for direct contact of MyD88 with the IL1 receptor complex subunit, IL1RaCp, which is indispensable for IL1β signaling (26). Alternatively, these interactive sites within MyD88 could participate in IL1β-induced oligomerization of MyD88 through homotypic interactions mediated by its TIR domain.

To sort out these possibilities, a second series of mutagenesis experiments was conducted. Five selected mutations were engineered in full-length MyD88 to allow a comparative analysis of its direct interaction with IL1RaCp (Table II). HEK 293T cells were co-transfected with Myc-IL1RaCp and AU1-MyD88 or its mutant constructs. We used immunoprecipitation followed by Western blotting to assess the effect of mutated residues on the receptor TIR-adaptor TIR interaction. As demonstrated in Fig. 5, only MyD88 D195A/R196A/D197A mutant showed loss of binding to IL1RaCp, whereas other mutants, including MyD88 P200H, retained their ability to bind IL1RaCp. This selective loss of receptor binding function by the MyD88 D195A/R196A/D197A mutant led us to develop the
three-dimensional docking model of MyD88 and IL1RAcP and verify the strategic position of these three residues as a main interactive site on the surface of the MyD88 TIR domain for its binding to the TIR domain of IL1RAcP.

The Development of the Three-dimensional Docking Model of MyD88-IL1RAcP Interaction—The three-dimensional docking model (Fig. 6) was developed by optimized superposition of two mutually interacting TIR domains of MyD88 and IL1RAcP. The negatively charged side of MyD88 TIR domain (see Fig. 2B) was selected as a possible interface of the molecule. This side contains Asp^{195}/Arg^{196}/Asp^{197} residues that are essential, on the basis of mutagenesis studies (Fig. 5), for MyD88-TIR heterotypic interaction with IL1RAcP-TIR. Then, a suitable positively charged site on the MyD88 TIR domain for its heterotypic interaction with IL1RAcP was determined by PSSHOW (SYBIL-Tripos software package). Separation surface indicates that there is no crossing of molecular surfaces, and the distance between them is within the range of 0.4–4.7 Å, whereas their topology is diverse and contains several deep pockets.

Development of this three-dimensional model allowed us to verify the contribution of the triplet of functionally important residues Asp^{195}/Arg^{196}/Asp^{197} to the binding reaction with IL1RAcP TIR domain. An analysis of the tertiary structure of two TIR domains that participate in the docking model indicates that three mutated residues (Asp^{195}/Arg^{196}/Asp^{197}), which are responsible for a loss of MyD88 binding to IL1RAcP, are involved in the interaction with residues 527–534 of IL1RAcP previously identified to play a key role in the IL1β signaling pathway (26, 27). Thus, our study identified a complementary site on the MyD88 TIR domain that contributes to its interaction with the IL1RAcP TIR domain. We therefore postulate that the negatively charged “knob,” partially composed of BB loop on the surface of the MyD88 TIR domain (Fig. 2B), fits into the positively charged lysine patch formed by residues 527, 530, and 532 of the IL1RAcP TIR domain previously identified by Radons et al. (26, 27) as essential for IL1β signaling.

Interactive Sites Involved in Oligomerization of MyD88 through Homotypic Interaction of Its TIR Domain—Following IL1β-induced interaction of IL1RAcP with MyD88, this adaptor oligomerizes and interacts with downstream signal transducers (2, 3, 7). Homotypic oligomerization of MyD88 due to its forced expression resulted in robust activation of NFκB reporter gene activity observed in the absence of IL1β stimulation. This receptor-independent effect of ectopically expressed MyD88 oligomers was abolished by co-transfected MyD88-TIR domain (data not shown). Therefore, we examined the direct interaction of full-length MyD88 co-expressed with MyD88 TIR domain or its mutants. We co-transfected HEK 293T cells with full-length MyD88 that contained c-Myc epitope tag along with the wild-type or mutated TIR domain that contained AU1 epitope tag. As demonstrated in Fig. 7, P200H mutant bound to full-length MyD88 to a similar extent as wild-type MyD88 TIR domain. However, mutations in Box 2 (D195A/R196A/D197A) and Box 3 (E-strand D275A and F285A/W286A) caused a loss of binding to MyD88, suggesting that these mutated residues constitute the interactive sites for homotypic oligomerization of the MyD88 TIR domain. Thus, the interacting site in Box 2 composed of residues Asp^{195}/Arg^{196}/Asp^{197} potentially has an additional function that may encroach on the ability of MyD88 to interact with IL1RAcP. However, in a cascade of signaling steps induced by IL1β, heterotypic interaction of IL1RAcP TIR domain with MyD88 TIR domain precedes oligomerization of MyD88. The latter depends on homotypic binding mediated by its TIR domain. Therefore, the interactive site in Box 3 is not likely to be involved in binding of MyD88 to IL1RAcP. Rather, this site participates in homotypic MyD88 oligomerization and possibly other transactions involving downstream signal transducers. This interpretation is consistent with the loss of inhibition of IL1β-induced signaling by the MyD88 TIR domain upon truncation of its carboxyl-terminal segment that contains Box 3.

Taken together, our results identify key residues in the MyD88 TIR domain that are responsible for its heterotypic interaction with IL1RAcP. In addition, we identified interactive sites for homotypic oligomerization of MyD88. These protein-protein interactions evoked by IL1β are essential for its signaling to the nucleus mediated by NFκB and other proinflammatory stress-responsive transcription factors. Mutations identified on the interacting surface of the MyD88 TIR domain are functionally important because they interfere with the induction of endogenous gene that encodes IL6. This inflammatory cytokine, along with IL1β, is responsible for cardinal signs of systemic inflammation: fever, leukocytosis, thrombocytosis, acute phase protein response, and tissue injury (1, 5). The development of the docking three-dimensional model of MyD88-IL1RAcP binding, in which a cluster of highly charged residues in Box 2 plays a key role, reaffirms their strategic role in contacting complementary site on IL1RAcP TIR domain. This site is composed of several positively
charged residues identified previously in EE loop residues 527–534 (26, 27). Thus, it is not surprising that the invariant Pro200 is inconsequential for MyD88-IL1RaCP interaction. However, canonical mutation of the invariant proline to histidine in TLR4, which abolishes its signaling by LPS (14), indicates that different ligands and their cognate TLRs utilize MyD88 in a structurally distinct way. In addition to TLR4, a proline to histidine mutation attenuated signaling mediated by TLR2, MAL, and IL1RaCP (14, 25, 26). We interpret the latter result as indicative of invariant proline playing a significant role in reshaping the EE loop in IL1RaCP or in interactions with the TIR domain of IL1RI or with signaling molecules other than MyD88.

In summary, our data indicate that following stimulation with IL1β/H9252, the MyD88 TIR domain binds to the IL1RaCP TIR domain via a highly charged interactive site composed of residues 195–197 within the BB loop of Box 2. We postulate, on the
basis of the three-dimensional docking model developed herein, that this interactive site is complementary to the previously identified site composed of residues 527–534 within the EE loop of the IL1RacP TIR domain (26, 27). Despite its proximity to the interactive site, invariant Pro200 of the MyD88 TIR domain does not play a role in IL1\textsubscript{H9252}-induced signaling. However, residues located in Box 3 are essential for subsequent homotypic interaction of MyD88 TIR domain. In a broader context, the results suggest that the IL1\textsubscript{H9252} signaling pathway differs from other ligand-initiated TLR intracellular signaling by usage of distinct structural motifs within the MyD88 TIR domain. Further mapping of the MyD88 surface will expand our understanding of its role in integrating signals derived from a variety of Toll-like receptors.

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