Mal Interacts with Tumor Necrosis Factor Receptor-associated Factor (TRAF)-6 to mediate NF-κB Activation by Toll-like Receptor (TLR)-2 and TLR4

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The Toll-interleukin-1 receptor domain-containing adapter Mal (MyD88 adapter-like protein) is involved in Toll-like receptor (TLR)-2 and TLR4 signal transduction. However, no studies have yet identified a function for Mal distinct from the related adapter MyD88. In this study, we have identified a putative TRAF6 interaction site in Mal but not in MyD88 and we demonstrate that Mal can be co-immunoprecipitated with TRAF6. Overexpression of MalE190A, which contains a mutation within the TRAF6-binding motif, failed to induce the expression of an NF-κB-dependent reporter gene, p65-mediated transactivation of gene expression, or activation of Jun N-terminal kinase or p42/p44 MAP kinase, which are induced with wild type Mal. MalE190A inhibited TLR2- and TLR4-mediated activation of NF-κB. These results identify a specific role for Mal in TLR-mediated signaling in regulating NF-κB-dependent gene transcription via its interaction with TRAF6.

MyD88 adapter-like protein (Mal) is also known as Toll/IL-1 receptor (TIR) domain-containing adapter protein (TIRAP).

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The abbreviations used are: Mal, MyD88 adapter-like protein; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; TRIF, TIR-containing adapter inducing interferon β; TRAM, TRIF-related adapter molecule; LPS, lipopolysaccharide; IL, interleukin; HEK, human embryonic kidney; pLC, poly(Lys-Lys); HA, hemagglutinin; jNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein. IKK, IL-1 receptor-associated kinase.

EXPERIMENTAL PROCEDURES
Cell Lines and Reagents—Human embryonic kidney (HEK) 293, HEK293T, and HEK293 stably transfected cells expressing TLR4 and MD2, were incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine and maintained in a 37 °C humidified atmosphere.

LPS K235 (Sigma) was repurified as described previously (19), Pam3Cys was obtained from EMC microcollections (Turbingen, Germany), poly(I)poly(C) (pLC) was obtained from Amersham Biosciences (Uppsala, Sweden), and anti-FLAG M2-horseradish peroxidase-conjugated antibody and anti-FLAG M2-agarose beads were from Sigma.

Plasmids—Full-length pDC304 Mal-HA, pDC304 Mal-HA N-terminal (14, 15), and pDC304 Mal-HA TIR domain (16, 17) have been described previously (1). MalE190A was generated using the QuikChange site-directed mutagenesis kit with pfu-Turbo (Stratagene, La Jolla, CA) using the pDC304 Mal-HA template. Gal4-p65(1–551) plasmid encoding the full-length p65 subunit of NF-κB fused to Gal4 DNA-binding do-
main was a kind gift from Lienhard Schmitz (German Cancer Research Centre, Heidelberg, Germany) (20). The Gal-luciferase reporter gene pFA-Jun and pFA-Erk-1 fusion vectors for analysis of JNK and P42/p44, respectively, were obtained from Stratagene (La Jolla, CA).

**Transient Transfections and Reporter Gene Assays—**HEK293 cells (2 × 10^6) were seeded in 96-well plates 24 h prior to transfection. Transfections were performed with FuGENE 6 (Roche Diagnostics). Mal vectors (0.5–2 ng) and b-luciferase were from Stratagene. NF-κB-dependent gene expression was determined using the 5 × b-luciferase reporter construct (Stratagene). Using the PathDetect transient transfection kit (Stratagene), co-transfection of pFR-luciferase in combination with Gal4-p65, pFA-Jun, or pFA-Erk-1 fusions, respectively, were used to analyze Mal/ MalE190A activation of MAP kinase and p65 transactivation. The Rous sarcoma virus β-galactosidase construct was used to normalize for transfection efficiency, and pRSV empty vector was used to maintain constant DNA. Cells were left untreated or treated with 100 ng/ml Pam3Cys, 50 ng/ml K235 LPS, or 25 μg/ml pL.C for 4 h where indicated. Transfected cells were lysed using Passive lysis buffer (Promega, Madison, WI) and assayed for luciferase and β-galactosidase assay reagent. Luminescence readings were corrected for Passive lysis buffer (Promega, Madison, WI) and assayed for luciferase and β-galactosidase activity using luciferase assay reagent (Promega) or β-galactosidase assay reagent. Luminescence readings were corrected for Passive lysis buffer (Promega, Madison, WI) and assayed for luciferase and β-galactosidase activity.

**Statistical Analysis—**Significance was evaluated using Student’s t test for unpaired data.

**Immunoprecipitation and Western Blot Analysis—**Immunoprecipitation and immunoblotting have been described previously (1). Western blot analysis was performed using anti-HA antibody (Rockland, Gilbertsville, PA) to detect Mal-Ha.

**RESULTS**

**Mal Associates with TRAF6—**Structural studies of TRAF6 in a complex with CD40 and TRANCE-R peptides suggested the structural determinant of the target protein contains a Pro-X-Glu-X-(aromatic/acidic residue) motif (18) for TRAF6 interaction. Analysis of the amino acid sequence of Mal indicated a putative TRAF6-binding domain at amino acid position 188–193 consisting of Pro-Pro-Glu-Leu-Arg-Phe similar to that described for IRAK and TRIF (Table I). Further analysis suggested that while Mal, TRIF, and TRAM all contain a putative TRAF6-binding motif, MyD88 does not, since the critical Glu residue (termed the P0 site) that has been found to confer specificity for TRAF6 interaction is changed to Ile in the corresponding MyD88 sequence (Table I).

To test the hypothesis that Mal was therefore able to interact with TRAF6, co-precipitation experiments were carried out in HEK293T cells transiently transfected with FLAG-tagged TRAF6 and HA-tagged Mal. As shown in Fig. 1A (lane 1), we were able to detect complexes containing Mal- and TRAF6-tagged proteins by co-immunoprecipitation. To evaluate the functional regions involved in this association, the ability of Mal N-terminal region (amino acids 1–74) and the Mal-TIR region (amino acids 74–235) were also assayed for their ability to co-precipitate with TRAF6. As can be observed in lanes 2 and 3, while the N-terminal region of Mal is unable to associate with TRAF6, the Mal-TIR domain, which harbors the putative TRAF6-binding motif, does co-immunoprecipitate with TRAF6. We next mutated the putative TRAF6-binding motif in Mal by changing the glutamic acid at position 190 to alanine and tested the ability of the mutant to interact with TRAF6. Surprisingly, TRAF6 was found to still co-immunoprecipitate with MalE190A (Fig. 1A, lane 4). The mutated form of Mal did, however, affect the ability of TRAF6 to signal (Figs. 1B, 2, and 3). As can be seen in Fig. 1B, while TRAF6 overexpression induced NF-κB-dependent luciferase expression, MalE190A inhibited this effect in a dose-dependent manner. Furthermore, MalE190A had no effect the activation of NF-κB by overexpressed IKK2 at the maximal dose required to inhibit TRAF6-mediated signaling. This result indicates that the critical Glu at position 190 in Mal is required for TRAF6 to induce downstream signaling events.

**The TRAF6-binding Motif in Mal Is Required for Mal to Signal—**Wild type Mal was able to induce an 8–11-fold increase in NF-κB-dependent luciferase expression in a DNA dose-dependent manner. MalE190A, however, failed to induce any significant luciferase expression over a corresponding DNA dose range (Fig. 2A). This result suggests that the TRAF6-binding motif is required to mediate NF-κB activation by Mal. Three further signals activated by Mal were similarly impaired by mutation of the TRAF6 interaction motif.

As can be seen in Fig. 2B, wild type Mal significantly drives p65-mediated transactivation in a dose-dependent manner. The maximal activation of 2.2-fold (p < 0.01) using this reporter system, although low, is consistent with the levels of activation previously reported in IL-1-mediated transactivation studies (20). Conversely, MalE190A failed to induce transactivation above control levels at comparable DNA concentrations to wild type Mal.

Mutant Mal was also unable to activate JNK or p42/p44 MAP kinase. As shown in Fig. 3C, overexpression of an optimal dose of plasmid encoding Mal (1 ng) was sufficient to drive JNK- and p42/p44-dependent luciferase expression (p = 0.02 and 0.002, respectively) at levels comparable with that which we have previously observed using these reporter assays (1, 21). MalE190A was unable to induce comparable luciferase expression above that of control levels at equivalent DNA concentrations. These data importantly demonstrate that Mal mediates the activation of the MAP kinases JNK and P42/P44 via its interaction with TRAF6.

**MalE190A Inhibits TLR-mediated Activation of NF-κB—**Finally, we addressed the role of Mal/TRAF6 interaction in TLR-mediated activation of NF-κB. HEK293 cells were transiently transfected with the NF-κB-linked luciferase reporter plasmid, plus TLR2, TLR3, or TLR4/MD2, rendering them responsive to the respective TLR ligands Pam3Cys, pI:C, and LPS. As can be seen in Fig. 3, MalE190A was able to specifically inhibit both TLR2- and TLR4-mediated activation of NF-κB-dependent luciferase expression in a dose-dependent manner. However, MalE190A was unable to inhibit TLR3-mediated activation of the NF-κB luciferase reporter, consistent with other studies demonstrating no role for Mal in TLR3-mediated signaling (7, 11). Taken together, these results indicate that the interaction between Mal and TRAF6 is necessary for the optimal induction of NF-κB-dependent gene expression, specifically upon TLR-2 and TLR4 stimulation.

**DISCUSSION**

In this study we have found a novel feature in Mal that distinguishes it from MyD88. Mal has a TRAF6 interaction
motif, which is required for Mal to signal. The motif is important for the functioning of Mal, since a mutant form of Mal, MalE190A, which contains a mutation of a critical amino acid within the TRAF6-binding motif failed to activate p65-mediated transactivation of gene expression, NF-κB-linked luciferase expression, and activation of JNK and p42/p44 MAP kinase. MyD88 does not contain a putative TRAF6-binding motif and links to TRAF6 via IRAK whose interaction with MyD88 is via homotypic death domain interaction (5). Importantly the mutated MalE190A also acted as a dominant negative toward TLR2- and TLR4-mediated activation of a NF-κB reporter gene. Taken together, these results demonstrate that the Mal-TRAF6 interaction is required for signal transduction by TLR2 and TLR4. Based on the novel data presented here, we propose a model whereby the role of Mal is to link TLR2 and TLR4 with TRAF6, independent of MyD88 and IRAK that may induce activation of the MAP kinase pathway and transactivation of the p65 subunit of NF-κB (Fig. 4).

Recent studies have shown that a corresponding point mutation, E252A, in the TRAF6-binding motif of TRIF abrogated TRIF/TRAF6 association (14), whereas our results indicated that MalE190A was still able to associate with TRAF6. There are, however, contradictions in the literature regarding the details of the TRIP/TRAF6 interaction and signaling (14, 15). Jiang et al. (14) found that TRIF E252A could no longer bind TRAF6, and overexpression of this mutant failed to drive NF-κB activation acting as a dominant negative in TLR3-mediated NF-κB activation (14). By contrast, Sato et al. (15) demonstrated a lack of association between TRIF and TRAF6 only by the E/A mutation of all three putative TRAF6-binding motifs in TRIF, combined with the truncation of the C terminus. Furthermore, these authors reported that the E252A mutant displayed only a limited inhibition of NF-κB activation (15), and the triple E/A TRIF mutations were required to inhibit TLR3-mediated NF-κB activation to a similar degree to that

(a) Mal associates with TRAF6. A, 2 × 10⁶ HEK293T cells were transiently co-transfected with FLAG-tagged TRAF6 and the indicated HA-tagged Mal constructs. Cells were co-immunoprecipitated with anti-FLAG M2-agarose beads, followed by Western blot analysis with anti-HA antibody. Cell extracts were used to check expression of transfected HA-Mal constructs and Flag-TRAF6 immunoprecipitation checked by immunoblotting with anti-FLAG M2-horseradish peroxidase-conjugated antibody (n = 3). B, HEK293 cells were transiently co-transfected with TRAF6 (1 ng), IKK2 (1 ng), and a range (0.5–5 ng) of MalE190A. Data represent relative stimulation of luciferase activity ± S.D. for triplicate determinations (*, p = 0.01) (n = 3).

(b) Mal interaction with TRAF6 mediates NF-κB activation and NF-κB transactivation. HEK293 cells (2 × 10⁶) were transiently co-transfected with κB-luciferase and β-galactosidase reporter plasmids in conjunction with Mal or MalE190A. Readings are normalized for each sample as expressed κB-luciferase over constitutively expressed β-galactosidase and plotted as fold stimulation. Results are ± S.D. for triplicate determinations (n = 3). B, HEK293 cells (2 × 10⁶) were co-transfected with the components of the p65-Gal4 system together with Mal and MalE190A plasmid. Results are ± S.D. for triplicate determinations (*, p = 0.01; **, p = 0.005) (n = 3). C, HEK293 cells (2 × 10⁶) were co-transfected with the components of the pFR-luciferase (80 ng) and c-jun (2 ng) and Elk-1 (5 ng) Gal4 fusion vectors, respectively, together with Mal (1 ng) and MalE190A (1 ng) plasmid. Results are ± S.D. for triplicate determinations (*, p = 0.05; **, p = 0.005) (n = 3).
potentiate NF-κB activation, inducing a stronger pro-inflammatory response typical of LPS. However, the total lack of cytokine expression in LPS-stimulated Mal-deficient macrophages (6, 7) suggests that the role of Mal is not simply to potentiate the canonical pathway in TLR4 signaling but to provide a separate and necessary signal, consistent with our results.

The mutant Mal was unable to drive several signals. A notable signal was p65-mediated transactivation of gene expression. This is the first demonstration of Mal activating this process, and it is possible that one explanation for the lack of NF-κB-dependent gene expression in Mal-deficient cells, despite NF-κB activation being relatively normal, is that the signal for p65-mediated transactivation requires Mal. While the primary means of regulating NF-κB activity is its sequestering in the cytosol by IκB, the transactivation of NF-κB to control gene expression is of critical importance to regulating the pro-inflammatory response (22). Our study implies that the interaction between Mal and TRAF6 is necessary for this transactivation.

In conclusion, we demonstrate Mal as a TRAF6 interacting protein. The ability to manipulate the interaction between Mal and TRAF6 may provide drug targets to control these signaling pathways, and thus NF-κB-dependent gene expression, while not interfering with the canonical pathway of the remaining TLRs, which are all dependent on MyD88.

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A Specific Role for Mal in TLR Signaling

FIG. 3. MalE190A inhibits TLR2- and TLR4-mediated NF-κB activation. Cells (2 × 10⁶) were co-transfected with luciferase and β-galactosidase reporter plasmids, in conjunction with a concentration range of MalE190A (0.5–2 ng, respectively, for HEK293 TLR2- and TLR4-transfected cells and 1–25 ng, respectively, for TLR4/MD2 stably transfected HEK293 cells). Cells were stimulated with LPS (10 ng/ml), Pam3Cys (100 ng/ml), or pIC (25 µg/ml) where indicated for 6 h. Data represent relative stimulation of luciferase activity ± S.D. for triplicate determinations (*, p < 0.01; **, p < 0.009) (n = 3).

FIG. 4. Model of Mal-mediated signaling via TRAF6 (see text).

demonstrated by Jiang et al. (14). The need to truncate the C terminus to inhibit association and NF-κB activation suggested that the C-terminal of an adapter such as TRIF may affect TRAF6 association and NF-κB activation. Our observation of the continued association of MalE190A and TRAF6 suggests a similar mechanism whereby other regions of Mal effect TRAF6 association that is not required for signal transduction.

Another explanation for the binding of Mal and TRAF6 in the absence of the P₁ Glu is that the two flanking P₂ and P₃ amino acids of MalE190A are still able to engage TRAF6 in our experimental conditions via hydrogen bonding, as demonstrated by Ye et al. (18) for TRANCE-R and TRAF6. However, the absence of the critical P₀ Glu interacting with TRAF6 renders TRAF6 unable to signal. While our study suggests that Mal interaction with TRAF6 is direct, there remains the possibility that Mal interaction with IRAK² may also lead to indirect interaction with TRAF6 as is the case with MyD88. The ability of Mal to interact with TRAF6 may serve to recruit additional TRAF6 molecules to the signaling complex, additive to that recruited by the MyD88/IRAK complex. This would

² A. Dunne and L. A. O’Neill, personal communication.