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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From the †Centre for Functional Genomics and Human Disease, Monash Institute of Reproduction and Development, Monash University, Melbourne, Victoria 3168, Australia and the ‡Cooperative Research Centre for Chronic Inflammatory Diseases, Department of Biochemistry and Biotechnology Institute, Trinity College, Dublin 2, Ireland

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)† (also known as Toll/IL-1 receptor (TIR) domain-containing adapter protein (TIRAP)) (1, 2) is a member of the family of the TIR domain-containing adapter proteins involved in Toll-like receptor (TLR) signaling (3, 4). MyD88 was the first adapter in the family to be described, and it plays a role in signal transduction by all TLRS, with the exception of TLR3 (5). Mal was found as a homologue of MyD88 (1, 2). In terms of function, it resembles MyD88 in that it is involved in the early activation of NF-κB and MAP kinases, but its use is restricted to signaling by TLR2 and TLR4 (6, 7). Two further adapters have been found to play a role in TLR signaling, TRIF (TLR-containing adapter inducing interferon β or TICAM-1) (8–10) is necessary for TLR3- and TLR4-mediated activation of NF-κB and another transcription factor IRF3, while TRAM (TRIF-related adapter molecule, also termed TICAM-2) (11–13) is essential for TLR4 signals, including IRF3. LPS-stimulated macrophages from MyD88- and Mal-deficient mice both displayed similar absence of cytokine production and delayed NF-κB activation but were normal for IRF3 activation and interferon β production. A double knock-out suggested that neither protein could compensate for the other. While a role for TRIF and TRAM in the IRF3 pathway distinguishes them from MyD88 and Mal, there is still no functional distinction between MyD88 and Mal in terms of function.

Recently, two groups reported the identification and functional role TRIF association with TRAF6 via a TRAF6-binding motif (14, 15). TRIF was found to mediate TLR3-induced activation of NF-κB via an association with TRAF6, independent of MyD88 and IRAK (15). The TRAF6 interaction motif was based upon work by Pullen et al. (16, 17) who first identified a cytoplasmic region of CD40 required to facilitate its binding of the TRAF-C domain of TRAF6. This interaction region was further defined by the elucidation of the crystal structure of the TRAF-C domain of TRAF6 in complex with peptides corresponding to CD40 or TRANCE-R (18). Structural analysis identified a nominal TRAF6-binding motif consisting of Pro-X-Glu-X-aromatic/acidic amino acids. Furthermore, three TRAF6-binding motifs were identified in IRAK, two in IRAK-2, one in IRAK-M, and one in RIP2 (see Table I). Sequential mutation of a critical glutamic acid in one, two, or all three TRAF6-binding motifs in IRAK proportionally attenuated IL-1-induced NF-κB activation.

In this study we have found that similar to TRIF, Mal has a putative TRAF6-binding motif. The motif is required for Mal to drive signals for NF-κB activation and the interaction between Mal and TRAF6 for downstream signaling events. This provides a distinguishing feature between Mal and MyD88 in TLR2- and TLR4-mediated responses.

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 K325 (Sigma) was reconstituted as described previously (19). Pam3Cys was obtained from EMC microcollections (Turbingen, Germany), poly(I:poly(C) (pIC) 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 (11–74), and pDC304 Mal-HA TIR domain (74–235) have been described previously (1). MalE190A was generated using the QuickChange 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 pGAL-Jun and pGAL-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 × 10⁵) 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 pβ-luciferase were from Stratagene. NF-κB-dependent gene expression was determined using the 5× β-luciferase reporter construct (Stratagene). Using the PathDetect transient transfection kit (Stratagene), co-transfection of pFR-luciferase in combination with Gal4-p65, pGAL-Jun, or pGAL-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 pLc for 4 h where indicated. Transfected cells were lysed using Passive lysis buffer (Promega, Madison, WI) and assayed for luciferase and β-galactosidase activity using luciferase assay reagent. NF-κB-mediated transactivation in a 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.

**TABLE I**

Putative TRAF6-binding motifs in human TIR domain-containing adapters

<table>
<thead>
<tr>
<th>TRAF6 Binding motif</th>
<th>PxxExxAArc/Ac</th>
<th>PxxERLAy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mal (188-193)</td>
<td>PXXERLAy</td>
<td>PXXERLAy</td>
</tr>
<tr>
<td>TRIF (250-255)</td>
<td>PXXERLAy</td>
<td>PXXERLAy</td>
</tr>
<tr>
<td>TRAM (181-186)</td>
<td>PXXERLAy</td>
<td>PXXERLAy</td>
</tr>
<tr>
<td>IRAK (1) (542-547)</td>
<td>PXXERLAy</td>
<td>PXXERLAy</td>
</tr>
<tr>
<td>IRAK (2) (585-596)</td>
<td>PxxExxAArc/Ac</td>
<td>PxxExxAArc/Ac</td>
</tr>
<tr>
<td>IRAK (3) (504-509)</td>
<td>PXXERLAy</td>
<td>PXXERLAy</td>
</tr>
<tr>
<td>IRAK-2 (1) (576-581)</td>
<td>PXXERLAy</td>
<td>PXXERLAy</td>
</tr>
<tr>
<td>IRAK-2 (2) (657-662)</td>
<td>PXXERLAy</td>
<td>PXXERLAy</td>
</tr>
<tr>
<td>IRAK-M (573-583)</td>
<td>PxxExxAArc/Ac</td>
<td>PxxExxAArc/Ac</td>
</tr>
<tr>
<td>RIP2 (194-199)</td>
<td>PXXERLAy</td>
<td>PXXERLAy</td>
</tr>
<tr>
<td>MyD88 (255-260)</td>
<td>PxxExxAArc/Ac</td>
<td>PxxExxAArc/Ac</td>
</tr>
</tbody>
</table>

Putative TRAF6-binding motifs were identified as described by Ye et al. (18) as PxxExxaromatic/acidic acid. The functional TRAF6-binding motif for TRIF has been described previously (14, 15).

**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 at position 190 in Mal is required for TRAF6 interaction (Table I). Further luciferase expression, specifically upon TLR-2 and TLR4-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 in NF-κB-dependent luciferase expression in a DNA dose-dependent manner. MalE190A also failed to drive 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.
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 TRIP 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...
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 IkB, 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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REFERENCES
