The Scaffold MyD88 Acts to Couple Protein Kinase Cε to Toll-like Receptors*

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Mice lacking protein kinase Cε (PKCε) are hypersensitive to both Gram-positive and Gram-negative bacterial infections; however, the mechanism of PKCε coupling to the Toll-like receptors (TLRs), responsible for pathogen detection, is poorly understood. Here we sought to investigate the mechanism of PKCε involvement in TLR signaling and found that PKCε is recruited to TLR4 and phosphorylated on two recently identified sites in response to lipopolysaccharide (LPS) stimulation. Phosphorylation at both of these sites (Ser-346 and Ser-368) resulted in PKCε binding to 14-3-3ε. LPS-induced PKCε phosphorylation, 14-3-3ε binding, and recruitment to TLR4 were all dependent on expression of the scaffold protein MyD88. In mouse embryonic fibroblasts and activated macrophages from MyD88 knockout mice, LPS-stimulated PKCε phosphorylation was reduced compared with wild type cells. Acute knockdown of MyD88 in LPS-responsive 293 cells also resulted in complete loss of Ser-346 phosphorylation and TLR4/PKCε association. By contrast, MyD88 overexpression in 293 cells resulted in constitutive phosphorylation of PKCε. A general role for MyD88 was evidenced by the finding that phosphorylation of PKCε was induced by the activation of all TLRs tested that signal through MyD88 (i.e. all except TLR3) both in RAW cells and in primary human macrophages. Functionally, it is established that phosphorylation of PKCε at these two sites is required for TLR4- and TLR2-induced NFκB reporter activation and IκB degradation in reconstituted PKCε−/− cells. This study therefore identifies the scaffold protein MyD88 as the link coupling TLRs to PKCε recruitment, phosphorylation, and downstream signaling.

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‡¶1 The abbreviations used are: TLR, Toll-like receptor; PKC, protein kinase C; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; WT, wild type; MEF, mouse embryonic fibroblasts; GFP, green fluorescent protein; IRF, interferon regulatory factor; TIR, Toll-IL1-R; GST, glutathione S-transferase; FCS, fetal calf serum; DMEM, Dulbecco’s modified Eagle’s medium; siRNA, short interfering RNA; YFP, yellow fluorescent protein; RANTES, regulated on activation normal T cell expressed and secreted; TRIF, Toll-IL1-R domain-containing adaptor inducing interferon-β; TRAM, TRIF-related adaptor molecule; PIP2, phosphatidylinositol 4,5-bisphosphate; LTA, lipoteichoic acid.
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ing, PKCα is involved in LPS- and poly(I-C)-induced NF-IL6 (19) and IRF3 (20) activation, respectively. LPS-induced MAPK activation, tumor necrosis factor-α production, and NFκB activation were shown to be PKCε-dependent in different cell types (21–23). Similarly, involvement of PKCε in MKP-1 and IL12 induction in response to LPS stimulation in macrophages and dendritic cells, respectively, has been demonstrated (24, 25). Despite many studies implicating PKC isoforms in TLR signaling, there is little evidence on the mechanism of their involvement. Recently, TRAM has been identified as a substrate of PKCε, and its phosphorylation has been shown to regulate RANTES production through IRF3 activation (26). However, the mechanism of PKCε activation in response to LPS remains obscure, and although TRAM phosphorylation by PKCε was required for it to signal, the exact function of this phosphorylation remains elusive. Similarly, Kubo-Murai et al. (27) have shown recently that PKCδ binds to Mal, and this binding promotes TLR2 and TLR4 signaling to p38 MAPK and IκB.

We have recently identified novel phosphorylation sites (Ser-346 and Ser-368) in the V3 region of PKCε that regulate its association with 14-3-3.3 Phosphorylation at these sites occurs sequentially through p38 (Ser-350) followed by GSK-3β (Ser-346) and auto-phosphorylation or classic PKC trans-phosphorylation (Ser-368) (43). The subsequent binding of 14-3-3β is required for efficient separation of cells at the end of cytokinesis. Here we investigated the role of PKCε in TLR signaling and discovered that LPS induced both recruitment of PKCε to TLR4 and its phosphorylation at Ser-346 and Ser-368, resulting in its association with 14-3-3β. PKCε recruitment to TLR4, phosphorylation, and binding to 14-3-3β were all dependent on MyD88 expression. We therefore propose that MyD88 represents the missing link that couples PKCε to TLR4 in response to LPS.

EXPERIMENTAL PROCEDURES

Reagents—Lipopolysaccharide (L7261) and lipoteichoic acid (L2515) were purchased from Sigma, and other TLR ligands were from Invivogen. All the inhibitors except BIRB 796 (a gift from Dr. Ana Cuenda, Dundee, Scotland, UK) were from Calbiochem. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies, ECL reagent, glutathione-Sepharose, and protein G-Sepharose were from Amersham Biosciences. The dual luciferase reporter system was purchased from Promega. Rabbit polyclonal antibodies against PKCε (C-15) and MyD88 (H8F-296) were from Santa Cruz Biotechnology. Mouse monoclonal anti-FLAG-M2 antibodies were obtained from Sigma. Mouse monoclonal Anti-GFP antibodies 3E1 (for Western blot) and 4E12/8 (for immunoprecipitation) were from the London Research Institute monoclonal facility. Rabbit polyclonal anti-phospho-p38 antibodies were from Cell Signaling. Generation of phospho-specific antibodies to serine 346 and serine 368 were carried out essentially as described previously (28) using the immunogens DRSKS(P)APTS and KITNS(P)GQRR, respectively. All other reagents were from Sigma.

Plasmids—GFP-PKCe WT, GFP-PKCe regulatory domain, Myc PKCe WT, and PKCe mutants in pEGFP-C1 and in pCDNA4 TO vectors were constructed by PCR and subcloning and were sequence-verified. The PKCe regulatory domain construct was cloned into the pEGFP-C1 vector. The human MyD88 construct was provided by Dr. Shizu Akira. The cDNA was re-cloned into pCDNA 3.1 and pCMV 2B vectors by PCR cloning and then sequence-verified. GST-14-3-3-β was from Professor Alastair Aitken. FLAG- and YFP-tagged TLR (TLR2, -3, and -4) constructs were a kind gift from Professor Golenbock. IRF3-dependent luciferase reporter construct, pGRL-3561 (29), was a kind gift from Dr. Ganes Sen.

Cells and Transfections—293 cells stably expressing human TLR4, MD2, and CD14 (referred to as 293/hTLR4) were purchased from Invivogen. These cells were maintained in Dulbeccos modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS), 10 μg/ml blasticidin, and 50 μg/ml Hygromycin at 37°C in a humidified chamber with 5% CO2. RAW 264.7 cells were maintained in DMEM with 10% FCS at 37°C in a humidified chamber with 10% CO2. PKCε+/–/– mouse embryonic fibroblasts (MEFs) have been described earlier (30), whereas MEFs from MyD88–/– mice were isolated from 12-day-old embryos and maintained in DMEM with 10% FCS. Peripheral blood monocytes were isolated by elutriation as described elsewhere (31). Monocytes were differentiated into macrophages for 3 days using 100 ng/ml recombinant human macrophage colony-stimulating factor (Peprotech) in RPMI 1640 medium containing 5% FCS and 100 units/ml penicillin/streptomycin. 293/hTLR4 cells were transfected at ~80% confluency with Lipofectamine 2000 or LTX (Invitrogen) to the manufacturer’s instructions. For NFκB reporter activation assays, cells were transfected with NFκB-TA-Luc (Clontech) and pHL-Renilla (Promega) at 1:10 ratio using Lipofectamine LTX.

Generation of Stable Cell Lines—~70% confluent RAW cells in 10-cm plates were transfected with 10 μg/plate of the plasmid DNA (GFP-PKCe WT, Ser-346/S368A, S368A, or vector control) using Lipofectamine 2000. Cells were split into 15-cm plates the next day and selected with 500 μg/ml Zeocin (Invitrogen) or 1 mg/ml G418 (depending on the constructs). Single clones were picked, and the rest were pooled and analyzed for GFP-PKCe expression by Western blot.

siRNA Knockdown—MyD88-N siRNA duplex (Qiagen) targeted the N-terminal region (nucleotides 181–201) of human MyD88 and had the following sequence 5′-CCGGCAACUGAAAGGAGA3′. 293/hTLR4 cells in 6-well plates were transfected with 50 nM siRNA using 5 μl of Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. 48 h after transfection with siRNA, cells were retransfected with GFP-PKCe. For experiments investigating PKCe-TLR4 interaction after MyD88 knockdown, 60-mm plates were used, and 48 h after MyD88 siRNA transfection cells were co-transfected with GFP-PKCe and FLAG-TLR4. Cells were analyzed after a further 24 h.

3 A. T. Saurin, J. Durgan, A. J. Cameron, A. Faisal, M. S. Marber, and P. J. Parker (2008) Nat. Cell. Biol., in press.
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**RESULTS**

**LPS Triggers Phosphorylation of PKCe**—The defective innate immunity of PKCe knock-out mice formally defines PKCe as a key regulator of TLR responses. For Gram-negative bacteria the relevant signaling paradigm involves LPS stimulation of TLR4; however, there is little direct evidence that TLR4 activation engages PKCe. Recent studies have identified two phosphorylation sites within the V3 region of PKCe that enable its binding to 14-3-3β and that are associated with the engagement of PKCe in signaling processes. To determine whether LPS activation of TLR4 triggers these responses, RAW 264.7 cells (here referred to as RAW cells) stably expressing GFP-PKCe were treated with LPS, and phosphorylation of PKCe was determined by Western blot using phospho-specific antibodies. Phosphorylation of both the Ser-346 and Ser-368 sites was found to be induced in response to LPS (Fig. 1A); a similar induction was also seen in GFP-PKCe-transfected 293 cells stably expressing the human proteins TLR4, MD2, and CD14 (Fig. 1B). There is a degree of constitutive phosphorylation of PKCe at the Ser-368 site in both cell types; however, there is clear LPS-dependent induction. LPS also induced Ser-346 phosphorylation of GFP-PKCe in HEK 293 cells transiently expressing TLR4/MD2; however, no phosphorylation was observed in vector control transfected cells (data not shown). To test the
response of the endogenous PKCe in terms of phosphorylation at Ser-346 and Ser-368, use was made of the 14-3-3β interaction of Ser-346/368 doubly phosphorylated PKCe (see below). Although this assay does not distinguish whether both sites are induced for the endogenous protein, in light of the observations above it is anticipated that both are increased (serum titers for the site-specific antisera were found to be inadequate for Western blotting the endogenous protein). Pulldown with GST-14-3-3 demonstrated that LPS induced an increased recovery of PKCe for both ectopic GFP-PKCe and the endogenous PKCe (Fig. 1C). A similar pulldown assay was used to demonstrate LPS-induced PKCe phosphorylation in primary human macrophages (Fig. 1D). The absolute requirement for dual 346/368 phosphorylation for the 14-3-3β interaction was confirmed through the use of single and double Ser → Ala mutants (Fig. 1E).

The LPS-induced responses in RAW cells expressing GFP-PKCe were time- and dose-dependent (Fig. 1, F and G). For both the Ser-346 and Ser-368 sites the responses peak at 20–30 min, and both are effectively at basal levels by 2 h. As illustrated (Fig. 1F), the induction of Ser-346 and Ser-368 was >7- and >2-fold, respectively. Over a series of experiments the mean induction for Ser-346 was 7 ± 1.01- and 2.5 ± 0.16-fold for Ser-368. Consistent with the mechanism of Ser-346 phosphorylation in fibroblasts (Ser-350 phosphorylation by p38 primes Ser-346 for phosphorylation by GSK3-β; see below), activation site phosphorylation of p38 MAPK was induced and peaked just ahead of PKCe Ser-346 phosphorylation. After 30 min of stimulation, phosphorylation of Ser-346 was induced by as little as 10 ng/ml of LPS and was maximum at 25 ng/ml.

**LPS Induces PKCe Phosphorylation via p38α/β and GSK-3**—Previous studies in fibroblasts have identified Ser-346 as a target for GSK-3, primed by p38α/β phosphorylation at Ser-350. To determine whether these same pathways account for the LPS-induced response in macrophages, cells were stimulated with LPS and various inhibitors assessed for their effects on Ser-346 phosphorylation. Three different GSK-3 inhibitors were found to block LPS-induced Ser-346 phosphorylation (Fig. 2A and data not shown for SB216763). Inhibition of all four p38 MAPK isoforms (with BIRB 796 (32)) also blocked Ser-346 phosphorylation indicative of a priming role (Fig. 2B). However, the p38α/β-selective inhibitors failed to block LPS-induced PKCe Ser-346 phosphorylation (see further below). Previously, p38α/β have been implicated in UV-induced PKCe Ser-346 phosphorylation in fibroblasts. Hence the effect of LPS was investigated in MEFs expressing GFP-PKCe. As in RAW cells, pan-p38 MAPK inhibition blocked LPS-induced Ser-346 phosphorylation, whereas the p38α/β-selective inhibitors (SB203580 and SB202190) were not inhibitory (Fig. 2C). To confirm the specificity of this behavior, the UV response was re-assessed in MEFs in parallel to the LPS response. As shown in Fig. 2D, BIRB inhibited both the LPS- and UV-induced phosphorylation of PKCe at the Ser-346 site. However, whereas SB203580 inhibited the UV-induced response, it did not inhibit the LPS-induced response. These distinct patterns of behavior are indicative of selective activation and/or targeting of specific

**FIGURE 2. LPS-induced PKCe Ser-346 phosphorylation depends on p38α/β and GSK3β.** A, GSK-3β inhibitors inhibit LPS-induced Ser-346 phosphorylation. RAW cells expressing GFP-PKCe were pretreated with SB415286 (30 μM) or lithium (20 mM) for 15 min and then treated with LPS (200 ng/ml) for 20 or 50 min. Cells were lysed, and equal amounts of total cellular proteins were resolved by LDS-PAGE and immunoblotted with anti-Ser(P)-346 and anti-GFP antibodies. B, inhibition of p38 blocks LPS-induced Ser-346 phosphorylation. RAW cells expressing GFP-PKCe were pretreated with BIRB 796 (10 μM) for 15 min and then stimulated with LPS (200 ng/ml) for 20 and 50 min. Cell lysates were analyzed by immunoblotting as described above. C, LPS-induced Ser-346 phosphorylation is not inhibited by p38α/β-specific inhibitors. PKCe expressing MEFs reconstituted with GFP-PKCe were pretreated with BIRB 796 (10 μM), SB203580 (10 μM), SB202190 (10 μM), or lithium (20 mM) for 15 min and then treated with LPS (200 ng/ml) for 60 min. Cells were lysed, and equal amounts of total cell lysates were immunoprecipitated (IP) with anti-GFP antibodies. Immunoprecipitated proteins were analyzed by immunoblotting as described above. D, p38α/β inhibitors selectively inhibit UV- and not LPS-induced Ser-346 phosphorylation. Reconstituted MEFs were pretreated with BIRB 796 (10 μM) or SB203580 (10 μM) for 15 min followed by LPS (200 ng/ml) treatment or UV (100 J/m²) exposure for 60 min. GFP-PKCe was immunoprecipitated with anti-GFP antibodies and analyzed as described above. E, LPS- and UV-induced Ser-368 phosphorylation is not inhibited by BIMI. RAW cells stably expressing GFP-PKCe were pretreated with BIMI (2 or 10 μM) for 15 min followed by LPS (200 ng/ml) or UV (100 J/m²) for 10 μg/ml treatment for 30 min. GFP-PKCe was immunoprecipitated from cell lysates with anti-GFP antibodies and phosphorylation status analyzed with Ser(P)-346-specific antibodies.
FIGURE 3. PKCe Ser-346 phosphorylation by different TLRs. A, TLR2 ligands induce Ser-346 phosphorylation. RAW cells expressing GFP-PKCε were stimulated with LTA (10 μg/ml) or FSL-1 (1 μg/ml) for 20, 30, or 60 min. Cells were lysed, immunoprecipitated with anti-GFP antibodies, and analyzed by immunoblotting using anti-Ser(P)-346, anti-Ser(P)-368, and anti-GFP antibodies. B, all TLR ligands except for TLR3 induce Ser-346 phosphorylation. RAW cells expressing GFP-PKCε were stimulated with LPS (200 ng/ml), LTA (10 μg/ml), Pam3CSK4 (1 μg/ml), flagellin (10 μg/ml), single strand RNA (10 μg/ml), or ODN1826 (10 μg/ml). Cells were lysed, and cell lysates were immunoprecipitated (IP) with anti-GFP antibodies. Immunoprecipitates were analyzed by immunoblotting with anti-Ser-346 and GFP antibodies. C, TLR4 but not TLR3 induces Ser-346 phosphorylation in 293 cells. FLAG-TLR4 and FLAG-TLR3 were overexpressed in 293/hTLR4 cells. Cells were stimulated with LPS (200 ng/ml) or poly(I-C) (10 μg/ml) for 60 min and lysed, and cell lysates were analyzed for Ser-346 phosphorylation and TLR expression by immunoblotting. D, 293/hTLR4 cells overexpressing F-TLR3 respond to poly(I-C). 293/hTLR4 cells were co-transfected with F-TLR4 or F-TLR3 along with an IRF-3-dependent reporter (pGL3–561) and Renilla control overnight. Cells were then stimulated with LPS (200 ng/ml) or poly(I-C) (10 μg/ml) for 6 h. IRF-3 reporter activation was analyzed by the dual luciferase assay system according to the manufacturer's instructions. E, PKCe phosphorylation by different TLR ligands in primary human macrophages. Human macrophages, differentiated from monocytes as described under “Experimental Procedures,” were treated with 200 ng/ml LPS, 10 μg/ml LTA, 20 μg/ml poly(I-C), or 100 ng/ml flagellin for 50 min. Cells were lysed, and cell lysates were pulled down with GST-14-3-3β overnignt and blotted with anti-PKCε antibodies. Total cell lysates were analyzed for protein loading, and the membrane from pull-down was stained with Coomassie for GST-14-3-3β loading.

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p38 MAPKs to the same priming phosphorylation of PKCe in response to distinct agonists.

To assess the expected PKCe dependence for Ser-368 phosphorylation, LPS- or LTA (TLR2 ligand; see further below)-stimulated cells were pretreated with BIMI (classic PKC + novel PKC inhibitor). Unexpectedly, no inhibition of Ser-368 phosphorylation was observed even at high BIMI concentration (Fig. 2E), although there was an inhibition by Go6976, a novel PKC inhibitor (data not shown) indicating that a distinct basophilic protein kinase is involved in the LPS response. Thus, although LPS triggers the phosphorylation of the 14-3-3β-binding sites of PKCe in both macrophages and fibroblasts, it does so via p38α/β + GSK-3 alongside an unknown basophilic kinase. This contrasts with the p38α/β + GSK-3 and PKC-dependent phosphorylation of these sites under other conditions. Nevertheless, this notable distinction, it is evident that LPS/TLR4 induce phosphorylation of PKCe in line with the established in vivo requirement for this kinase.

Multiple TLR Ligands Trigger PKCe Phosphorylation—PKCe knock-out mice are defective in the clearance of both Gram-positive and Gram-negative bacteria. To determine whether model Gram-positive ligands acting via TLR2 promote PKCe phosphorylation as determined above for LPS-TLR4, RAW cells were stimulated with either LTA or FSL-1 (TLR2/TLR6). Both ligands were found to increase PKCe Ser-346 and Ser-368 phosphorylation (Fig. 3A), although the Ser-368 site was delayed relative to the Ser-346 site and induced only a 2-fold increase. In view of the conservation of this response, ligands engaging TLRs 1–9 were tested (Fig. 3A). All but the TLR3 ligand poly(I-C) stimulated PKCe Ser-346 phosphorylation. To ensure that poly(I-C) was acting via TLR3, 293/hTLR4 cells were transfected with TLR3 or TLR4, and responses to poly(I-C) and LPS were monitored. LPS-induced phosphorylation of...
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**A** 14-3-3 Pull down

|          | WT            | MyD88-/- |
|----------|---------------|----------|
| PKCε     |               |          |
| MyD88    |               |          |
| GST-14-3-3 |               |          |
| Mouse Embryo Fibroblasts (MEFs) | | |

**B**

|          | WT            | MyD88-/- |
|----------|---------------|----------|
| TPA/Calyculin LPS 200 ng/ml | | |
| PKCε     |               |          |
| MyD88    |               |          |
| GST-14-3-3 |               |          |
| PKCε     |               |          |

**C**

|          | LPS 200 ng/ml | P-S346 | GFP-PKCε |
|----------|---------------|--------|----------|
| WT       |               |        |          |
| MyD88   |               |        |          |

**D**

|          | LPS 200 ng/ml | P-S346 | GFP-PKCε |
|----------|---------------|--------|----------|
| MyD-N siRNA |             |        |          |

**E**

|          | LPS 200 ng/ml | P-S346 | GFP-PKCε |
|----------|---------------|--------|----------|
| pCMV     |               |        |          |
| F-MyD88  |               |        |          |

**F**

|          | LPS 200 ng/ml | P-S346 | GFP-PKCε |
|----------|---------------|--------|----------|
| pCDNA    |               |        |          |
| MyD88    |               |        |          |

**FIGURE 4.** MyD88-dependent PKCe phosphorylation. A, LPS-induced PKCe binding to GST-14-3-3β in WT and not MyD88 -/- MEFs. WT and MyD88 -/- MEFs were stimulated with LPS (200 ng/ml) and lysed. Equal amounts of protein were pulled down with GST-14-3-3β and analyzed with anti-PKCε and MyD88 antibodies. The membrane from the pull-down was stained with Coomassie as a GST-14-3-3 loading control. B, LPS-induced PKCe binding to 14-3-3β in thioglycollate elicited peritoneal macrophages from WT and not MyD88 -/- mice. Thioglycollate-elicited macrophages were isolated from WT and MyD88 -/- mice as described under “Experimental Procedures” and cultured in 10-cm plates. Cells were stimulated with LPS (200 ng/ml) or TPA (400 nm)/calyculin (10 nm) as indicated, and PKCe/GST-14-3-3β binding in extracts was analyzed as described above. C, MyD88 knockdown inhibits Ser-346 phosphorylation in 293/TLR4 cells. MyD88 and control siRNA were transfected in 293/TLR4 cells as described under “Experimental Procedures.” 48 h after transfections, cells were transfected again with GFP-PKCε and stimulated with LPS (200 ng/ml) 24 h later. Cells were lysed, and cell lysates were analyzed by immunoblotting using anti-Ser(P)-346, anti-GFP, and anti-MyD88 antibodies. D, re-expression of MyD88 rescues the effect of MyD88 knockdown on Ser-346 phosphorylation. MyD88 was knocked down in 293/TLR4 cells by MyD88-N siRNA transfection, and cells were co-transfected after 48 h with vector or FLAG-tagged mouse MyD88 (F-m-MyD88) and GFP-PKCε. Cells were analyzed 24 h later and analyzed for Ser-346 phosphorylation and expression of FLAG-tagged mouse MyD88 and GFP-PKCε by immunoblotting using specific antibodies. E and F, MyD88 overexpression-induced Ser-346 phosphorylation. MyD88 knockdown MyD88 (E), MyD88 (F), or the respective empty vectors were co-transfected with GFP-PKCε in 293/TLR4 cells. Cells were stimulated with LPS (200 ng/ml) for 60 min and lysed. Cell lysates were analyzed for GFP-PKCε phosphorylation as described earlier.

Ser-346 in TLR4 expressing cells was observed, but no such response to poly(I-C) in TLR3-expressing cells was seen (Fig. 3C). However, poly(I-C) did induce an IRF-3-dependent reporter response in TLR3-expressing cells but not in cells expressing TLR4 (Fig. 3D). Thus a functionally linked TLR3 receptor is not linked to PKCe phosphorylation.

A similar PKCe response was observed for primary human macrophages. In freshly isolated macrophages challenged with LPS, LTA, poly(I-C), or flagellin, an increase in PKCe phosphorylation at both Ser-346 and Ser-368 was observed with all ligands except poly(I-C) (TLR3) as indicated by PKCe binding to GST-14-3-3β (Fig. 3E). The induction observed for LTA was ~2-fold compared with the 4–6-fold induction for LPS and flagellin. As noted above these responses are indicative of ligand-induced PKCe phosphorylation at 346/368 sites, although we cannot distinguish the changes in the individual sites by this procedure.

**MyD88 Links PKCe to TLRs**—The pattern of PKCe responses to these TLRs parallels their engagement of MyD88, i.e. all but TLR3. To test whether MyD88 was responsible for linking PKCe to TLRs, cells from MyD88 knock-out mice were tested for responses to LPS using capture on GST-14-3-3β beads to monitor endogenous PKCe Ser-346/Ser-368 phosphorylation as evidenced by the increased recovery of PKCe complexed to 14-3-3β. By contrast to MyD88-replete MEFs, no response to LPS was observed in MyD88 knock-out cells (Fig. 4A). Peritoneally elicited macrophages from WT mice also responded to LPS with a substantial increase in PKCe recovered in a 14-3-3β pull-down, equivalent to that observed with the potent combination of TPA/calyculin. By contrast there was no LPS-induced recovery of PKCe from the MyD88 knock-out macrophages, despite a “WT” response to TPA/calyculin (Fig. 4B).

The evidence from the knock-out model demonstrates a requirement for MyD88 in the TLR4-triggered PKCe response. To confirm this in an acute model, siRNAs to MyD88 were employed to knock down MyD88 expression. As illustrated in Fig. 4C, siRNA knockdown of MyD88 also abrogated the LPS-induced PKCe phosphorylation.
Ser-346 phosphorylation. The specificity of the effect of MyD88 knockdown on Ser-346 phosphorylation was confirmed by rescue experiments with mouse MyD88. As shown in Fig. 4D, re-expression of mouse MyD88 in cells with knockdown of endogenous MyD88 by human-specific siRNA rescued the Ser-346 phosphorylation. Interestingly, when FLAG-tagged MyD88 or untagged MyD88 is overexpressed in 293/hTLR4 cells, constitutively high Ser-346 phosphorylation is observed with no further increase in LPS stimulation (Figs. 4, E and F). This reflects elevated p38 phosphorylation in these MyD88 overexpressing cells (data not shown).

PKCe Is Complexed with TLR4 via MyD88—The adaptor role of MyD88 and its requirement for linking PKCe to TLR4 suggested that PKCe may be physically associated with the (active) receptor. By employing FLAG-tagged TLR4, it was found that a fraction of co-expressed GFP-PKCe or myc-PKCe (Fig. 5A) could be recovered in TLR4 immunoprecipitates in an LPS-inducible manner; much lower levels of PKCe could be recovered in TLR4 immunoprecipitates (data not shown and see Fig. 5B). The complex formation between TLR4 and PKCe was further supported by co-immunoprecipitation of YFP-TLR4 and myc-PKCe using both anti-GFP (YFP-TLR4) and anti-Myc antibodies (data not shown). To map the PKCe domain required for its recruitment to TLR4, GFP-PKCe regulatory and GST-PKCe catalytic domains were used. GFP-PKCe regulatory domain was recovered in FLAG-TLR4 immunoprecipitates from 293/hTLR4 cells (Fig. 5B), whereas GST-PKCe catalytic domain could not be recovered with FLAG-TLR4 (data not shown). The constitutive basal recovery of PKCe in TLR4 immunocomplexes was enhanced by co-expression of MyD88 (Fig. 5C). siRNAs to MyD88 were employed to determine its requirement in the PKCe-TLR4 interaction. FLAG-TLR4 interaction with GFP-PKCe, determined by immunoprecipitation as described above, was reduced in 293/hTLR4 cells transfected with MyD88 siRNA as compared with the control siRNA (Fig. 5D). We further confirmed this by a rescue experiment, in which endogenous MyD88 was knocked down in 293/hTLR4 cells and myc-PKCe and YFP-TLR4 interaction was determined by immunoprecipitation in the presence of vector control or mouse MyD88 (Fig. 5E). The effect of MyD88 knockdown on PKCe-TLR4 interaction was completely recovered by expression of ectopic mouse MyD88.

Phosphorylation of PKCe at the 14-3-3β-Binding Sites Is Required for NFκB Transcriptional Activation—To assess the role of PKCe phosphorylation downstream of TLRs, stable cell lines expressing matched amounts of WT GFP-PKCe and an S346A/S368A PKCe mutant were tested for activation of a luciferase NFκB reporter. WT PKCe expression enhanced LTA- and LPS-induced luciferase expression. By contrast, expression of the S346A/S368A PKCe mutant failed to facilitate luciferase expression (Fig. 6A). To ensure that this distinction was not an artifact of the clonal isolates, pools of stably expressing cells were also tested. As observed for the clonal isolates, the WT protein supported induction of luciferase, whereas the mutant did not (Fig. 6B).

To determine whether the effects of PKCe WT expression were exerted through the control of IκB degradation, WT and mutant PKCe were compared for their ability to support LTA-induced IκB degradation. Although the WT protein was effective in supporting IκB degradation in response to LTA, the mutant was not (Fig. 6C).

**DISCUSSION**

Activated macrophages play an integral part in launching an effective immune response to microbial infections (33). Studies using specific inhibitors and dominant interfering mutants of
different isoforms have identified PKCs as important mediators of endotoxin signaling in macrophages (19, 23, 25, 34). Previously we showed that targeted disruption of the PKCe gene in mice leads to compromised innate immunity (18). PKCe−/− mice had defects in clearance of both Gram-positive (TLR2) and Gram-negative (TLR4) bacterial infections. LPS-induced signaling in macrophages from these mice was severely attenuated resulting in reduced production of pro-inflammatory cytokines. Here we sought to investigate the mechanism of PKCe coupling to the TLR and found it to be phosphorylated and in a complex with TLR4 upon LPS stimulation. PKCe recruitment to TLR4 and the phosphorylation that resulted in its ability to bind 14-3-3β were both MyD88-dependent. The requirement for the recruitment of PKCe and its subsequent phosphorylation downstream of TLRs were evidenced by the effects of the WT and mutant PKCe in supporting NFκB-dependent luciferase expression.

Most of the studies implicating PKCe in TLR signaling have analyzed its function in response to LPS (TLR4) apart from in vivo studies with Gram-positive (TLR2) and Gram-negative (TLR4) bacteria (18). Recently, McGettrick et al. (26) have demonstrated that IRF3 activation and RANTES production in response to LPS and not poly(I-C) is regulated by PKCe phosphorylation of TRAM. Because TRAM works as a bridging adaptor for TRIF in the MyD88-independent pathway downstream of TLR4, this might imply a restricted role for PKCe in MyD88-independent TLR4 signaling (35, 36). The evidence here, however, indicates that PKCe gets phosphorylated in response to activation of TLRs that signal through MyD88 in RAW cells and primary human macrophages (Fig. 3, B and E), thereby implying a broader role in MyD88-dependent pathways. Ligands for TLR4 and TLR2/6 induced Ser-346 and Ser-368 phosphorylation that resulted in PKCe association with 14-3-3β in vitro (Figs. 1, A and B, and 3A). PKCe phosphorylation and the subsequent 14-3-3β binding capacity elicited by TLR4 activation were MyD88-dependent, and the evidence for this dependence was provided by three different approaches. (a) PKCe binding to GST-14-3-3β in MyD88 knock-out MEFs and thioglcollate-elicited macrophages in response to LPS was compromised compared with their WT counterparts (Fig. 4, A and B). (b) Knockdown of MyD88 significantly reduced LPS-induced PKCe phosphorylation at Ser-346 and Ser-368 (Fig. 4, C and D). (c) Overexpression of MyD88 in 293/hTLR4 cells induced ligand-independent phosphorylation of PKCe and recovery on 14-3-3β beads (Fig. 4, E and F, and data not shown). Because PKCe binding to 14-3-3β requires its phosphorylation at both Ser-346 and Ser-368 (Fig. 1E), LPS-induced recovery of PKCe by 14-3-3β from RAW cells, primary human macrophages, mouse embryo fibroblasts, and thioglcollate-elicited macrophages (Figs. 1, 3, and 4) demonstrated that the endogenous protein behaved like the ectopic PKCe and was therefore phosphorylated at both of these required sites and that one or both sites were induced in response to ligand. So LPS-induced PKCe phosphorylation in a variety of cell lines (including immortalized and primary cells) suggests a conserved role of PKCe in TLR4 signaling. LPS also induced recruitment of a small fraction of PKCe to TLR4. PKCe complexed with TLR4 was phosphorylated on Ser-346 (data not shown). Similar to its phosphorylation, PKCe interaction with TLR4 was also dependent on MyD88, which we therefore can conclude recruits the kinase to TLR4. Consistent with this conclusion, overexpression of MyD88-induced ligand-independent binding of PKCe to TLR4 and MyD88 knockdown reduced this association in a manner rescued by re-expression of an siRNA-resistant MyD88 (Fig. 5, C, D, and E).

Several studies have shown phospholipase C activation and diacylglycerol production in response to LPS (37–39). Therefore, it follows that diacylglycerol production after LPS stimulation might be important in PKCe recruitment and activation. A PIP2 binding domain has been identified in the bridging adaptor Mal/TIRAP that recruits it to the membrane upon LPS
stimulation (40). Mal then delivers MyD88 to TLR4 for further downstream signaling. As noted above, PKCε is recruited to the activated TLR4 by MyD88 (Fig. 5). Because MyD88 recruitment to TLR4 itself is mediated by Mal and is PIP2-dependent, PKCε recruitment to TLR4 might also be dependent on Mal and PIP2. However, PKCε phosphorylation was observed in response to several TLRs, including TLR7 and TLR9, which are localized to intracellular compartments potentially less rich in PIP2, suggesting a Mal and PIP2-independent recruitment of PKCε. The implication is that MyD88 plays the dominant role in this response.

Two different phosphoserine/threonine-containing motifs have been identified in 14-3-3-binding proteins (41). The Ser-346 site (RSKSAP) is within an optimal 14-3-3 binding mode I (RSX(pS/T)XP) motif, whereas the sequence surrounding Ser-368 (RKALSDFX) resembles a mode II (RXX(pS/T)XP) motif, although lacking a proline at the +2 position. Recently we have characterized the 14-3-3-PKCε dimer binding in detail and demonstrated that the 14-3-3β dimer binds to PKCε phosphorylated at both Ser-346 and Ser-368 in a 1:1 dimer-PKCε complex.3 Phosphorylation of both sites is also required for 14-3-3β binding to PKCε after LPS stimulation, and mutation of either or both of these sites to alanine abolishes this binding (Fig. 1). Therefore, the 14-3-3β binding resulting from the phosphorylation at Ser-346 and Ser-368 downstream of TLR4/MyD88 is tightly regulated by different inputs. Downstream of TLR4 these inputs come from p38α/β (priming phosphorylation of Ser-350), GSK-3β (Ser-346), and an unknown basophilic kinase (Ser-368). LPS-induced phosphorylation of p38 precedes Ser-346 phosphorylation (Fig. 1F), supporting its priming role for Ser-346 phosphorylation by GSK-3β. Using pan-p38 and p38α/β-specific inhibitors (BIRB 796 and SB203580, respectively), we demonstrated that, depending on the signal, different p38 isoforms can prime PKCε for Ser-346 phosphorylation in the same cell model, indicative of selective activation and/or targeting of p38 family members by different agonists (Fig. 2). For LPS-induced priming, p38α/β was involved in both RAW cells and MEFs. Ser-368 was expected to be an auto-phosphorylation site; however, BIM I did not inhibit the phosphorylation in response to LPS, although it was able to inhibit the response to UV. This ruled out auto-phosphorylation and/or trans-phosphorylation by classical or novel PKC isoforms. A classical PKC inhibitor Go6976 did, however, very efficiently inhibit LPS-induced Ser-368 phosphorylation (Fig. 2E). Considering no inhibition by BIM I, this effect of Go6976 indicates that a non-PKC Go6976-sensitive kinase is involved.

14-3-3 binding can regulate various properties of its target proteins, including localization, stability, activity, and/or interactions with other proteins (42). We therefore tested the possible role of 14-3-3 binding in PKCε recruitment to TLR4. However, there was no difference in TLR4 recruitment of WT or the S346A/S368A mutant PKCε in response to LPS, thereby excluding a targeting role for this complex (data not shown).14-3-3β binding locks PKCε in an open conformation,3 thereby regulating its lipid-independent activity. We propose that a similar mechanism of lipid-independent activation of PKCε by 14-3-3β binding exists in response to TLR activation. However, it remains to be determined whether a 14-3-3β-binding defective mutant of PKCε gets activated in response to LPS or not.

In conclusion, we have shown that PKCε is linked to various TLRs through the adaptor protein MyD88. This serves both to recruit the kinase to the receptor and to enable its phosphorylation at previously defined 14-3-3β-binding sites. These events are shown to be critical for the subsequent degradation of IκB and activation of NFκB. This novel mechanism of receptor coupling to PKCε therefore underlies signaling downstream of TLRs, which explains the compromised innate immune response in PKCε knock-out mice.

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