THE SCAFFOLD MYD88 ACTS TO COUPLE PROTEIN KINASE C EPSILON (PKCε) 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 (S346 and S368) resulted in PKCε binding to 14-3-3β. In response to LPS-induced PKCε phosphorylation, 14-3-3β binding and recruitment to TLR4 were all dependent on expression of the scaffold protein MyD88. In mouse embryo fibroblasts and activated macrophages from MyD88 knockout mice, LPS-stimulated PKCε phosphorylation was reduced compared to WT cells. Acute knockdown of MyD88 in LPS responsive 293 cells also resulted in complete loss of S346 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 IkB 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.

INTRODUCTION

Toll like receptors (TLRs) recognize microbial pathogen associated molecular patterns (PAMPs) and initiate common signaling pathways leading to specific inflammatory responses through activation of transcription factors such as nuclear factor κB (NFκB) and interferon regulatory factors (IRFs) (1). TLRs signal through Toll-IL1-R (TIR) domain-containing adaptor proteins that are recruited to receptor TIR domains upon ligand binding (2). Of five TIR domain-containing adaptors identified in humans, MyD88 has been shown to be involved in signal transduction for all TLRs except TLR3 (3,4). MyD88 deficiency in macrophages and dendritic cells (DCs) leads to loss of MAPK activation, NFκB activation and proinflammatory cytokine production in response to various TLR ligands (5-7). However, some responses downstream of TLR4 are either only delayed (NFκB and MAPK activation) or not affected (INFβ production) in MyD88 deficient cells (8). These constitute MyD88-independent pathways and have lead to the identification of...
other adaptor proteins. MyD88 adaptor-like (Mal) and Toll-IL-1R domain-containing adaptor inducing IFN-beta (TRIF)-related adapter molecule (TRAM) work as bridging adaptors for MyD88 and TRIF to activate NFκB and IRF3 respectively. Mal/MyD88 signal from TLR2/TLR4 to regulate NFκB activation (MyD88-dependent) while TRAM/TRIF signal to IRF3 in response to TLR4 activation (MyD88-independent) (9). The specificity in the activation of transcription factors by different TLRs using common signaling pathways is therefore achieved by differential use of adaptor proteins.

Protein Kinase C (PKC) is a family of closely related serine/threonine kinases that regulate diverse cellular processes such as proliferation, survival, immunity and apoptosis (10,11). Based on the cofactor requirements the PKC family is classified into three subfamilies; conventional PKCs (α, βI, βII and γ) regulated by diacylglycerol, phosphatidylserine and calcium, novel PKCs (δ, ε, θ and η) regulated by diacylglycerol and phosphatidylserine, and atypical PKCs (ζ and λ/ι) that do not require diacylglycerol for activation (12). Studies in mice lacking different PKC isoforms have established an important role for PKCs in intracellular immune signaling (reviewed (13)). PKCβ knockout mice have an immunodeficiency due to defective B cell activation (14), while PKCζ is required for TCR-mediated T-cell activation (15). Mice deficient in PKCδ and PKCζ have defective B cell energy and NFκB signaling, respectively (16,17). PKCε−/− mice have impaired innate immunity and fail to clear Gram-positive and Gram-negative bacterial infection (18). LPS and TNFα induced nitric oxide, TNFα, IL1β and prostaglandin E2 (PGE2) production in PKCε−/− macrophages is reduced and this is attributed to the impaired NFκB activation upstream of IκB kinase β (IKKβ). Other PKC isoforms have also been implicated in TLR signaling. PKCα is involved in LPS- and poly I:C-induced NF-κB (19) and IRF3 (20) activation, respectively. LPS-induced MAPK activation, TNFα production and NFκB activation were shown to be PKCζ-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 PKCe and its phosphorylation has been shown to regulate RANTES production through IRF3 activation (26). However, the mechanism of PKCe activation in response to LPS remains obscure and although TRAM phosphorylation by PKCe was required for it to signal, the exact function of this phosphorylation remains elusive. Similarly, Kubo-Murai et al have shown recently that PKCδ binds to Mal and this binding promotes TLR2 and TLR4 signaling to p38 MAPK and IκB (27).

We have recently identified novel phosphorylation sites (S346 and S368) in the V3 region of PKCe that regulate its association with 14-3-3β (Saurin et al submitted). Phosphorylation at these sites occurs sequentially through p38 (S350) followed by GSK-3β (S346) and auto-phosphorylation or cPKC trans-phosphorylation (S368). 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 PKCe in TLR signaling and discovered that LPS induced both recruitment of PKCe to TLR4 and its phosphorylation at S346 and S368, resulting in its association with 14-3-3β. PKCe 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 PKCe to TLR4 in response to LPS.

**EXPERIMENTAL PROCEDURES**

*Reagents:* Lipopolysaccharide (L7261) and Lipoteichoic acid (L2515) were purchased from Sigma, while other TLR ligands were from Invivogen. All the inhibitors except BIRB 796 (a gift from Dr. Ana Cuenda, Dundee) 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 PKCe (C-15) and MyD88 (HFL-296) were from Santa Cruz. Mouse monoclonal Anti-Flag-
M2 antibodies were obtained from Sigma. Mouse monoclonal Anti-GFP antibodies 3E1 (for Western) 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-PKCε WT, GFP-PKCε Regulatory domain, Myc PKCε WT and PKCε mutants in pEGFP-C1 and in pCDNA4/TO vectors were constructed by PCR, subcloning and were sequence verified. The PKCε 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, pGL3-561 (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 Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS), 10µg/ml blasticidin and 50µg/ml HygroGold at 37°C in a humified chamber with 5% CO₂. RAW 264.7 cells were maintained in DMEM with 10% FCS at 37°C in a humified chamber with 10 % CO₂. PKCεΔ mouse embryo fibroblasts (MEFs) have been described earlier (30), while MEFs from MyD88Δ 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 100ng/ml recombinant human M-CSF (Peprotech, UK) in RPMI 1640 containing 5%FCS and 100 U/ml penicillin/streptomycin. 293/hTLR4 cells were transfected at ~80% confluency with Lipofectamine 2000 or LTX (Invitrogen) according to the manufacturer’s instructions. For NFκB reporter activation assays, cells were transfected with NFκB-TA-Luc (Clonetech) and phRL-Renilla (Promega) at a 10:1 ratio using Lipofectamine LTX.

**Generation of stable cell lines:** ~70% confluent RAW cells in 10 cm plates were transfected with 10µg/plate of the plasmid DNA (GFP-PKCε WT, S346/S368A, S346A, S368A or vector control) using Lipofectamine 2000. Cells were split into 15 cm plates on the next day and selected with 500µg/ml of zeocin (Invitrogen) or 1mg/ml G418 (depending on the constructs). Single clones were picked and the rest were pooled and analysed for GFP-PKCε expression by western. PKCε MEFs stably expressing different GFP-PKCε constructs were generated by transfection and zeocin selection of polyclonal populations (Saurin unpublished).

**siRNA knockdown:** MyD88-N siRNA duplex (Qiagen) targeted the N-terminal region (nucleotides 181 to 201) of human MyD88 and had the following sequence 5'-CCGGCAACUGGAGACACAdTdT-3' and 5'-UUGUGUCUCCAGUUGCCGdAdT-3'. 2 x 10⁵ 293/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 hours after transfection with siRNA, cells were re-transfected with GFP-PKCε. For experiments investigating PKCε-TLR4 interaction after MyD88 knockdown, 60mm plates were used and 48 hours after MyD88 siRNA transfection cells were co-transfected with GFP-PKCε and Flag-TLR4. Cells were analysed after a further 24 hours.

**Immunoprecipitation, pulldown and western blot analysis:** Cell lysis was carried out on ice with lysis buffer containing 1% NP40, 50mM Tris HCl pH 7.4, 120mM NaCl, 5mM sodium vanadate, 50mM sodium fluoride and EDTA free protease inhibitor tablet from Roche. Protein concentration was measured and equal amounts of protein were immunoprecipitated by incubation at 4°C with anti-GFP and anti-Flag antibodies as indicated for 2h.
followed by a further 1h incubation with protein G Sepharose. Beads were washed twice with TNET (TNE + 1% Triton X-100), once with TNE (50 mM Tris-Cl, pH 7, 140 mM NaCl, and 5 mM EDTA) and boiled in LDS sample buffer. Proteins were resolved by 4-12% NUPAGE gels, transferred to PVDF membranes and immunoblotted with specific antibodies. GST-14-3-3β pulldown assays were performed using bacterially expressed GST-14-3-3β loaded glutathione beads. Native extracts were tumbled overnight at 4°C prior to washing twice with TNET (TNE + 1% Triton X-100), once with TNE (50 mM Tris-Cl, pH 7, 140 mM NaCl, and 5 mM EDTA) and elution in LDS buffer.

Isolation of thioglycollate-elicited peritoneal macrophages: Peritoneal macrophages were isolated as described previously (18). Briefly, mice (MyD88 KO and C57 black/6 WT) were injected with 3% thioglycollate intra-peritoneally and sacrificed after 4 days. The peritoneum was flushed with sterilized PBS (containing 5mM EDTA) and the peritoneal suspension containing macrophages was carefully removed and centrifuged at 200g for 10 min. Cells were resuspended and seeded in RPMI 1640 with 10% FCS in 10 cm plates at 37°C with 10% CO2. After 1 hour incubation, non-adherent cells were removed by excessive washing with PBS. Cells were used for experiments on the following day.

RESULTS

LPS triggers phosphorylation of PKCε.

The defective innate immunity of PKCε knock-out mice formally defines PKCε as a key regulator of TLR responses. For gram-negative bacteria the relevant signalling paradigm involves LPS stimulation of TLR4; however there is little direct evidence that TLR4 activation engages PKCε. Recent studies have identified two phosphorylation sites within the V3 region of PKCε which enable its binding to 14-3-3β and are associated with the engagement of PKCε in signalling processes (Saurin et al submitted). To determine whether LPS activation of TLR4 triggers these responses, RAW264.7 cells (here referred to as RAW cells) stably expressing GFP-PKCε were treated with LPS and phosphorylation of PKCε was determined by western blot using phospho-specific antibodies. Phosphorylation of both the S346 and S368 sites were found to be induced in response to LPS (Figure 1A); a similar induction was also seen in GFP-PKCε transfected 293 cells stably expressing the human proteins TLR4, MD2 and CD14 (Figure 1B). There is a degree of constitutive phosphorylation of PKCε at the S368 site in both cell types, however there is clear LPS-dependent induction. LPS also induced S346 phosphorylation of GFP-PKCε in HEK 293 cells transiently expressing TLR4/M2, however no phosphorylation was observed in vector control transfected cells (data not shown). To test the response of the endogenous PKCε in terms of phosphorylation at S346 and S368, use was made of the 14.3.3β interaction of S346/368 doubly phosphorylated PKCε (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 titres for the site-specific antisera were found to be inadequate for western blotting the endogenous protein). Pull down with GST-14.3.3β demonstrated that LPS induced an increased recovery of PKCε for both ectopic GFP-PKCε and the endogenous PKCε (Figure 1C). A similar pull down assay was used to demonstrate LPS-induced PKCε phosphorylation for the 14.3.3β interaction was confirmed through the use of single and double S>A mutants (Figure1E).

The LPS-induced responses in RAW cells expressing GFP-PKCε were time and dose dependent (Figure1 F,G). For both the S346 and S368 sites the responses peak at 20-30 minutes and both are effectively at basal levels by 2h. As illustrated (Figure 1F) the induction of S346 and S368 was >7 fold and >2 fold respectively. Over a series of experiments the mean induction for S346 was 7 ± 1.01 fold and 2.5 ± 0.16 fold for S368. Consistent with the mechanism of S346 phosphorylation in fibroblasts (S350 phosphorylation by p38 primes S346 for phosphorylation by GSK3-β; see below) activation site phosphorylation of p38 MAP kinase was induced and peaked just ahead of PKCε S346 phosphorylation. After 30 min of stimulation,
phosphorylation of S346 was induced by as little as 10ng/ml of LPS and was maximum at 25ng/ml.

**LPS induces PKCe phosphorylation via p38γ/δ and GSK-3.**

Previous studies in fibroblasts have identified S346 as a target for GSK-3, primed by p38α/β phosphorylation at S350. 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 S346 phosphorylation. Three different GSK-3 inhibitors were found to block LPS-induced S346 phosphorylation (Figure 2A and data not shown for SB216763). Inhibition of all four p38 MAP kinase isoforms (with BIRB 796; (32)) also blocked S346 phosphorylation indicative of a priming role (Figure 2B). However the p38α/β-selective inhibitors failed to block LPS-induced PKCe S346 phosphorylation (Data not shown; see further below). Previously p38α/β have been implicated in UV-induced PKCe S346 phosphorylation in fibroblasts (Saurin et al submitted). Hence the effect of LPS was investigated in mouse embryo fibroblasts (MEFs) expressing GFP-PKCe. As in RAW cells, pan-p38 MAP kinase inhibition blocked LPS-induced S346 phosphorylation, whilst the p38α/β-selective inhibitors (SB203580, SB202190) were not inhibitory (Figure 2C). To confirm the specificity of this behaviour the UV response was re-assessed in MEFs in parallel to the LPS response. As shown in Figure 2D, BIRB inhibited both the LPS- and UV-induced phosphorylation of PKCe at the S346 site. However, whilst SB203580 inhibited the UV-induced response, it did not inhibit the LPS-induced response. These distinct patterns of behaviour are indicative of selective activation and/or targeting of specific p38MAP kinases to the same priming phosphorylation of PKCe in response to distinct agonists.

To assess the expected PKCe dependence for S368 phosphorylation, LPS- or LTA-(TLR2 ligand; see further below) stimulated cells were pre-treated with BIMI (cPKCe+nPKCe inhibitor). Unexpectedly, no inhibition of S368 phosphorylation even at higher BIMI concentration was observed (Figure 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 p38γ/δ + 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 (Saurin et al submitted). Notwithstanding 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 S346 and S368 phosphorylation (Figure 3A) although the S368 site was delayed relative to the S346 site and induced only a ~2-fold increase. In view of the conservation of this response, ligands engaging TLRs 1-9 were tested (Figure 3B). All but the TLR3 ligand poly I:C stimulated PKCe S346 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 monitored. LPS induced phosphorylation of S346 in TLR4 expressing cells was observed, but no such response to poly I:C in TLR3 expressing cells was seen (Figure 3C). However, poly I:C did induce an IRF-3 dependent reporter response in TLR3 expressing cells but not in cells expressing TLR4 (Figure 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 S346 and S368 was observed with all ligands except poly I:C (TLR3) as indicated by PKCe binding to GST-14.3.3β (Figure 3E). The
induction observed for LTA was ~2-fold compared to 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 can not 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 S346/S368 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 (Figure 4A). Peritoneal 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 (Figure 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 Figure 4C siRNA knock-down of MyD88 also abrogated the LPS-induced PKCe S346 phosphorylation. The specificity of the effect of MyD88 knockdown on S346 phosphorylation was confirmed by rescue experiments with mouse MyD88. As shown in Figure 4D, re-expression of mouse MyD88 in cells with knockdown of endogenous MyD88 by human specific siRNA rescued the S346 phosphorylation. Interestingly, when Flag-tagged MyD88 or untagged MyD88 is overexpressed in 293/hTLR4 cells, constitutively high S346 phosphorylation is observed with no further increase on LPS stimulation (Figures 4E and F). This reflects elevated p38 phosphorylation in these MyD88 over-expressing 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. Employing Flag-tagged TLR4 it was found that a fraction of co-expressed GFP-PKCe or myc-PKCe (Figure 5A) could be recovered in TLR4 immunoprecipitates in an LPS inducible manner; much lower levels of PKCe were recovered in anti-Flag control immunoprecipitates (Data not shown and 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 (Figure 5B), while 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 (Figure 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 to the control siRNA (Figure 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 (Figure 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-PKCε and an S346/368A PKCε mutant were tested for activation of a luciferase NFκB reporter. WT PKCε expression enhanced LTA and LPS induced luciferase expression. By contrast expression of the S346/368A PKCε mutant failed to facilitate luciferase expression (Figure 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 while the mutant did not (Figure 6B).

To determine whether the effects of PKC WT expression were exerted through the control of IκB degradation WT and mutant PKCε were compared for their ability to support LTA-induced IκB degradation. While the WT protein was effective in supporting IκB degradation in response to LTA, the mutant was not (Figure 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 PKCε gene in mice lead to compromised innate immunity (18). PKCε−/− 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 PKCε coupling to the TLR and found it to be phosphorylated and in a complex with TLR4 upon LPS stimulation. PKCε 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 PKCε and its subsequent phosphorylation downstream of TLRs were evidenced by the effects of the WT and mutant PKCε in supporting NFκB dependent luciferase expression.

Most of the studies implicating PKCε in TLR signaling have analysed 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 have demonstrated that IRF3 activation and RANTES production in response to LPS and not poly I:C is regulated by PKCε phosphorylation of TRAM (26). Since TRAM works as a bridging adapter for TRIF in the MyD88-independent pathway downstream of TLR4, this might imply a restricted role for PKCε in MyD88-independent TLR4 signaling (35,36). The evidence here however, indicates that PKCε gets phosphorylated in response to activation of TLRs that signal through MyD88 in RAW cells and primary human macrophages (Figure 3B and 3E), thereby implying a broader role in MyD88-dependent pathways. Ligands for TLR4 and TLR2/6 induced S346 and S368 phosphorylation that resulted in PKCε association with 14-3-3β in vitro (Figures 1A, B and 3A). PKCε 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) PKCε binding to GST-14-3-3β in MyD88 knockout MEFs and thioglycollate elicited macrophages in response to LPS was compromised compared to their WT counterparts (Figure 4A and B); b) knockdown of MyD88 significantly reduced LPS-induced PKCε phosphorylation at S346 in 293/hTLR4 cells (Figure 4C and D); and c) overexpression of MyD88 in 293/hTLR4 cells induced ligand independent phosphorylation of PKCε and recovery on 14-3-3β beads (Figure 4E, F and data not shown). Since PKCε binding to 14-3-3β requires its phosphorylation at both S346 and S368 (Figure 1E), LPS-induced recovery of PKCε by 14-3-3β from RAW cells, primary human macrophages, mouse embryo fibroblasts and thioglycollate elicited macrophages (Figures 1, 3 and 4) demonstrated that the endogenous protein behaved like the ectopic PKCε 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 PKCε phosphorylation in a variety of cell lines (including immortalized and primary cells) suggests a conserved role of PKCε in TLR4 signaling. LPS also induced recruitment.
of a small fraction of PKCe to TLR4. PKCe complexed with TLR4 was phosphorylated on S346 (data not shown). Similar to its phosphorylation, PKCe interaction with TLR4 was also dependent on MyD88 which we can conclude therefore 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 (Figure 5C, D and E).

Several studies have shown phospholipase C activation and diacylglycerol production in response to LPS (37-39). It follows therefore that DAG 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, PKCe is recruited to the activated TLR4 by MyD88 (Figure 5). Since MyD88 recruitment to TLR4 itself is mediated by Mal and is PIP2 dependent, PKCe recruitment to TLR4 might also be dependent on Mal and PIP2. However, PKCe 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 PKCe. The implication is that MyD88 plays the dominant role in this response.

Two different phospho-serine/threonine containing motifs have been identified in 14-3-3 binding proteins (41). The S346 site (RSKSAP) is within an optimal 14-3-3 binding mode I (RSXpS/TXP) motif while the sequence surrounding S368 (RKALSFSD) resembles a mode II (RXXXXpS/TXP) motif although lacking a proline at the +2 position. Recently we have characterized the 14-3-3-PKCe binding in detail and demonstrated that the 14-3-3β dimer binds to PKCe phosphorylated at both S346 and S368 in a 1:1 dimer:PKCe complex (Saurin et al submitted). Phosphorylation of both sites is also required for 14-3-3β binding to PKCe after LPS stimulation and mutation of either or both of these sites to alanine abolishes this binding (Figure 1). Therefore, the 14-3-3β binding resulting from the phosphorylation at S346 and S368 downstream of TLR4/MyD88 is tightly regulated by different inputs. Downstream of TLR4 these inputs come from p38γ/δ (priming phosphorylation of S350), GSK-3β (S346), and an unknown basophilic kinase (S368). LPS-induced phosphorylation of p38 precedes S346 phosphorylation (Figure 1F), supporting its priming role for S346 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 PKCe for S346 phosphorylation in the same cell model, indicative of selective activation and/or targeting of p38 family members by different agonists (Figure 2). For LPS-induced priming p38γ/δ were involved in both RAW cells and MEFs. S368 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 S368 phosphorylation (Figure 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 PKCe recruitment to TLR4. However, there was no difference in TLR4 recruitment of WT or the S346A/S368A mutant PKCe in response to LPS thereby excluding a targeting role for this complex (data not shown). 14-3-3β binding locks PKCe in an open conformation (Saurin et al submitted), thereby regulating its lipid independent activity. We propose that a similar mechanism of lipid independent activation of PKCe 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 PKCe 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.

REFERENCES


FOOTNOTES

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**FIGURE LEGENDS**

**Figure 1:**

*LPS-induced PKCε phosphorylation and association with 14-3-3β.*

*A,* LPS-induced PKCε phosphorylation in RAW cells. RAW cells stably expressing GFP-PKCε were stimulated with 200ng/ml of LPS for 50 min. Cells were lysed, and cell lysates were either used directly (15µg, upper panel) or immunoprecipitated with anti-GFP antibodies (300µg, lower panel) and analysed by western blotting using anti-P-S346 and GFP antibodies (upper panel) or anti-P-S368 and GFP-antibodies (lower panel). *B,* LPS-induced PKCε phosphorylation in 293/hTLR4 cells. 293/hTLR4 cells transiently transfected with GFP-PKCε were stimulated with 200ng/ml LPS and analysed as described above. *C,* Phosphorylation and 14-3-3β binding of endogenous PKCε. PKCε from LPS stimulated RAW GFP-PKCε cells was pulled down with GST-14-3-3β (lower panel) and analysed by western using anti-PKCε antibodies. Membrane from pulldown was stained with comassie for GST-14-3-3β as loading control. *D,* PKCε phosphorylation in primary human macrophages. Human monocytes were differentiated into macrophages as described in “Methods” and stimulated with 200ng/ml LPS for times indicated. Cell lysates were pulled down with GST-14-3-3β and blotted with PKCε antibodies. *E,* Phosphorylation of both S346 and S368 is required for 14-3-3β binding. RAW cells stably expressing different mutants of GFP-PKCε were stimulated with 200ng/ml LPS for 50 min and a GST-14-3-3β pulldown assay was performed. Total cell lysates were probed with PKCε antibodies for equal expression in different clones. *F* and *G,* Time and concentration dependence of PKCε phosphorylation. GFP-PKCε expressing RAW cells were either stimulated with 200ng/ml of LPS for different times (*F*) or with different concentrations of LPS for 30 min (*G*). Cells lysates were immunoprecipitated and analysed for S346 and S368 phosphorylation using site specific antibodies.

**Figure 2:**

*LPS-induced PKCε S346 phosphorylation depends on p38γ/δ and GSK3β.*

*A,* GSK-3β inhibitors inhibit LPS-induced S346 phosphorylation. RAW cells expressing GFP-PKCε were pre-treated with SB415286 (30µM) or Li (20mM) for 15 min and then treated with LPS (200ng/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-PS346 and anti-GFP antibodies. *B,* Inhibition of p38 blocks LPS-induced S346 phosphorylation. RAW cells expressing GFP-PKCε were pre-treated with BIRB 796 (10µM) for 15 min and then stimulated with LPS (200ng/ml) for 20 and 50 min. Cell lysates were analysed by immunoblotting as described above. *C,* LPS-induced S346 phosphorylation is not inhibited by p38α/β specific inhibitors. PKCε-/- MEFs reconstituted with GFP-PKCε were pretreated with BIRB 796 (10µM), SB203580 (10µM) SB202190 (10µM), or Li (20mM) for 15 min and then treated with LPS (200ng/ml)
for 60 min. Cells were lysed and equal amounts of total cell lysates were immunoprecipitated with anti-GFP antibodies. Immunoprecipitated proteins were analysed by immunoblotting as described above. D, p38α/β inhibitors selectively inhibit UV- and not LPS-induced S346 phosphorylation. Reconstituted MEFs were pre-treated with BIRB 796 (10µM) or SB203580 (10µM) for 15 min followed by LPS (200ng/ml) treatment or UV (100 J/m²) exposure for 60 min. GFP-PKCe was immunoprecipitated with anti-GFP antibodies and analysed as described above. E, LPS- and LTA-induced S368 phosphorylation is not inhibited by BIMI. RAW cells stably expressing Flag-PKCe were pre-treated with BIM I (2µM or 10µM) or Go6976 (1µM) for 15 min followed by LPS (200ng/ml) or LTA (10µg/ml) treatment for 50 min. Flag-PKCe was immunoprecipitated from cell lysates with anti-Flag antibodies and phosphorylation status analysed with PS346 specific antibodies.

Figure 3:

PKCe S346 phosphorylation by different TLRs.
A. TLR2 ligands induce S346 phosphorylation. RAW cells expressing GFP-PKCe were stimulated with LTA (10µg/ml) or FSL-1 (1µg/ml) for 20, 30 or 60 minutes. Cells were lysed, immunoprecipitated with anti-GFP antibodies and analysed by immunoblotting using anti-PS346, anti-PS368 and anti-GFP antibodies. B. All TLR ligands except for TLR3 induce S346 phosphorylation. RAW cells expressing GFP-PKCe were stimulated with LPS (200ng/ml), LTA (10µg/ml), Pam3CSK4 (1µg/ml), FSL-1(100ng/ml), poly I:C (10µg/ml), Flagellin (10µg/ml), ssRNA (10µg/ml) or ODN1826 (10µg/ml). Cells were lysed and cell lysates were immunoprecipitated with anti-GFP antibodies. Immunoprecipitates were analysed by immunoblotting with anti-S346 and GFP-antibodies. C, TLR4 but not TLR3 induces S346 phosphorylation in 293 cells. Flag-TLR4 and Flag-TLR3 were overexpressed in 293/hTLR4 cells. Cells were stimulated with LPS (200ng/ml) or poly I:C (10µg/ml) for 60 min, lysed and cell lysates analysed for S346 phosphorylation and TLR expression by immunoblotting. D, 293hTLR4 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 (200ng/ml) or poly I:C (10µg/ml) for 6 hours. IRF-3 reporter activation was analysed by the dual luciferase assay system according to manufacturer’s instructions. E PKCe phosphorylation by different TLR ligands in primary human macrophages. Human macrophages, differentiated from the monocytes as described in “Methods”, were treated with 200ng/ml LPS, 10µg/ml LTA, 20µg/ml poly I:C or 100ng/ml Flagellin for 50 min. Cells were lysed and cell lysates were pulled down with GST-14-3-3β overnight and blotted with anti-PKCe antibodies. Total cell lysates were analysed for protein loading and the membrane from pull down was stained with comassie for GST-14-3-3β loading.

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 analysed with anti-PKCe and MyD88 antibodies. The membrane from the pulldown was stained with comassie 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 in materials and methods and cultured in 10 cm plates. Cells were stimulated with LPS (200ng/ml) or TPA (400nM)/calyculin (10nM) as indicated and PKCe/GST-14-3-3β binding in extracts was analysed as described above. C, MyD88 knockdown inhibits S346 phosphorylation in 293/hTLR4 cells. MyD88 and control siRNA were transfected in 293/hTLR4 cells as described in materials and methods. 48 hours after transfections, cells
were transfected again with GFP-PKCε and stimulated with LPS (200ng/ml) 24 hours later. Cells were lysed and cell lysates were analysed by immunoblotting using anti-PS346, anti-GFP and anti-MyD88 antibodies. D, Re-expression of MyD88 rescues the effect of MyD88 knockdown on S346 phosphorylation. MyD88 was knocked down in 293/hTLR4 cells by MyD88-N siRNA transfection and cells were co-transfected after 48 hours with vector or Flag-tagged mouse MyD88 (F-m-MyD88) and GFP-PKCε. Cells were analysed 24 hours later and analysed for S346 phosphorylation and expression of F-m-MyD88 and GFP-PKCε by immunoblotting using specific antibodies. E & F. MyD88 overexpression-induced S346 phosphorylation. Flag-MyD88 (E), MyD88 (F) or the respective empty vectors were co-transfected with GFP-PKCε in 293/hTLR. Cells were stimulated with LPS (200ng/ml) for 60 min and lysed. Cell lysates were analysed for GFP-PKCε phosphorylation as described earlier.

**Figure 5:**

*TLR4-PKCε association.*

A, PKCε co-immunoprecipitation with TLR4. Flag-TLR4 was co-transfected with GFP-PKCε or myc-PKCε in 293/hTRL4 cells. 24 hours after transfection, cells were stimulated with LPS (200ng/ml) for 50 min and cells were lysed. Cell lysates were immunoprecipitated with anti-Flag antibodies and immunoprecipitates were analysed by immunoblotting with anti-PKCε or anti-Flag antibodies. B, PKCε regulatory domain association with F-TLR4. 293/hTLR4 cells were co-transfected with F-TLR4 and GFP-PKCε, stimulated with LPS (200ng/ml) after 24 hours. Cells were lysed and lysates were analysed for PKCε-TLR4 association by co-immunoprecipitation as described above. C, MyD88 overexpression enhances TLR4-PKCε association. MyD88 or empty vector was co-transfected with GFP-PKCε and F-TLR4. 24 hours after transfection cells were stimulated with LPS (200ng/ml) for 50 min, lysed and cell lysates analysed for TLR4-PKCε association as described above. D, MyD88 knockdown suppresses TLR4-PKCε association. 293/hTLR4 cells were transfected with MyD88-N siRNA, followed 48 hours later by co-transfection of Flag-TLR4 and GFP-PKCε. Cells were stimulated 24 hours later with LPS, lysed and immunoprecipitated with anti-Flag antibodies. Immunoprecipitates were analysed by immunoblotting using GFP antibodies. E, Rescue of TLR4-PKCε association in MyD88 knockdown cells by re-expression of MyD88. MyD88 was knocked down in 293/hTLR4 cells by MyD88-N siRNA transfection and cells were co-transfected after 48 hours with YFP-TLR4, Myc-PKCε ± Flag-tagged mouse MyD88. Cells were analysed 24 hours later for TLR4-PKCε association by immunoprecipitation with anti-GFP antibodies and immunoblotting with PKCε, GFP and Flag antibodies.

**Figure 6:**

*PKCε phosphorylation is required for TLR2/4-induced NFκB activation.*

A, LPS and LTA-induced NFκB reporter activation in PKCε−/− MEF clones expressing vector, GFP-PKCε WT or GFP-PKCε AA. NFκB reporter and Renilla control plasmids were transfected in MEF clones at 10:1 ratio in 24 well plates in duplicates overnight. Cells were stimulated with 25ng/ml LPS or 10µg/ml LTA for 7 hours. NFκB reporter activation was analysed by the dual luciferase assay system according to manufacturer’s instructions. B, LPS and LTA-induced NFκB reporter activation in PKCε−/− MEF clones and pool cells expressing vector, GFP-PKCε WT or GFP-PKCε AA. Cells were transfected with NFκB and Renilla control plasmids, stimulated and analysed for reporter activation as described above. C, PKCε WT enhances IκB degradation in PKCε−/− MEFs in response to TLR2 activation. PKCε−/− MEF clones expressing vector, GFP-PKCε WT or GFP-PKCε AA were stimulated with 20µg/ml LTA for indicated times. Cells were collected and analysed for IκB degradation, PKCε expression and loading by western blot analysis using specific antibodies.
Figure 1:

A

RAW 264.7
LPS 200ng/ml
P-S346 ➔
GFP-PKCε ➔
Total Cell Lysate
0 50
0 50

B

293-hTLR4
0 50 Min.
0 50 Min.
P-S346 ➔
P-S368 ➔
GFP-PKCε ➔
Total Cell Lysate
Total Cell Lysate

C

LPS 200ng/ml
0 20 50 Min.
GFP-PKCε ➔
PKCε ➔
GST-14-3-3 ➔
GST-14-3-3 pull down
TCL

D

LPS 200ng/ml
PKCε ➔
GST-14-3-3 ➔
GST-14-3-3 pull down
Total cell lysate

E

LPS 200ng/ml
WT
AA
S346A
S368A
0 50
0 50
0 50
0 50
GFP-PKCε ➔
GST-14-3-3 ➔
GST-14-3-3 pull down
Total cell lysate

F

LPS 200ng/ml
0 5 10 20 30 60 90 120 Min.
P-S346 ➔
P-S368 ➔
GFP-PKCε ➔
P-p38 ➔

IP: Anti-GFP

G

LPS 30 Min
0 10 25 50 100 200 400 1000 ng/ml
P-S346 ➔
GFP-PKCε ➔

IP: Anti-GFP
Figure 2:

A

| Li 20mM | — | — | — | — | — | + | + |
| SB415286 30μM | — | — | — | + | + | — | — |
| LPS 200ng/ml | 0 | 20 | 50 | 20 | 50 | 20 | 50 |

GFP-PKCε-346 →
GFP-PKCε →
Total cell lysate

B

| BIRB 10μM | — | — | — | — | — | — | + |
| LPS 200ng/ml | 0 | 20 | 50 | 0 | 20 | 50 | Min. |

P346-GFP-PKCε →
GFP-PKCε →
Total Cell lysate RAW 264.7

C

| LPS 200ng/ml | 0 | 60 | 0 | 60 | 0 | 60 | 0 | 60 | 0 | 60 | 0 | 60 |
| SB | BIRB 203580 | BIRB 202190 | Li |

GFP-PS346 →
GFP-PKCε →
Reconstituted MEFs

IP: Anti-GFP

D

| SB203580 | LPS 200ng/ml | UV 100 J/m2 |
| BIRB | 0 | 60 | 60 | 60 | 0 | 60 | 60 | 60 | 0 | 60 | 60 |

P-S346 →
GFP-PKCε →
Reconstituted MEFs

E

| BIM I 10 μM | — | — | — | — | + | — | — |
| BIM I 2 μM | — | — | — | + | — | — | + |
| LTA 10μg/ml | 0 | 0 | 0 | 0 | 50 | 50 | 0 | 0 |
| LPS 200ng/ml | 0 | 50 | 50 | 50 | 0 | 0 | Min. |

P-S368 →
GFP-PKCε →
Figure 3:

A

\[
\begin{array}{c}
\text{IP: Anti-GFP} \\
\text{Total Cell Lysate}
\end{array}
\]

B

\[
\begin{array}{c}
\text{IP: Anti-GFP}
\end{array}
\]

C

\[
\begin{array}{c}
\text{IP: Anti Flag} \\
\text{Total Cell lysate}
\end{array}
\]

D

\[
\begin{array}{c}
\text{Relative IRF3 Reporter Activity}
\end{array}
\]

E

\[
\begin{array}{c}
\text{GST-14-3-3β pull down} \\
\text{Total Cell Lysate}
\end{array}
\]
Figure 4:

A 14-3-3 Pull down

LPS 200ng/ml

0 50 0 50 Min.

PKCε  

MyD88  

GST-14-3-3  

Total Cell lysate

Mouse Embryo Fibroblasts (MEFs)

B TPA/Calyculin

LPS 200ng/ml

0 50 0 50 Min.

PKCε  

GST-14-3-3β  

GST-14-3-3β Pull down

Total Cell lysate

C LPS 200ng/ml

0 60 0 60 Min.

P-S346  

GFP-PKCε  

Luc  

MyD N

D MyD-N siRNA

LPS 200ng/ml

P-S346  

GFP-PKCε  

Flag-m-MyD88

Vector  

F-m-MyD88

Min.

E LPS 200ng/ml

0 60 0 60 Min.

P-S346  

GFP-PKCε  

F-MyD88

pCMV  

F-MyD88

Min.

F LPS 200ng/ml

0 60 0 60 Min.

P-S346  

GFP-PKCε  

MyD88

pCDNA  

MyD88

Min.
Figure 5:

A

LPS 200ng/ml

Flag-TLR4

GFP-PKCε

Myc-PKCε

TCL

0 50 0 50

0 50 0 50

Min.

IP: Anti-Flag

Anti-Flag

B

Flag-TLR4

GFP-PKCε-R

LPS 200ng/ml

+ + – – + +

+ + + + – –

0 50 0 50 0 50

0 50

Min.

IP: Anti-Flag

Anti-GFP

C

LPS 200ng/ml

F-TLR4 + GFP-PKCε

pCDNA MyD88

0 60 0 60

0 60

Min.

IP: Anti-Flag

Total Cell Lysate

D

LPS 200ng/ml

Flag-TLR4 + GFP-PKCε

Control siRNA MyD88-N siRNA

0 60 0 60

0 60

Min.

IP: Anti-GFP

Total Cell lysate

E

Flag-mMyD88

LPS 200ng/ml

MyD88-N siRNA

0 60 0 60

0 60

Min.

IP: Anti-GFP

Flag-mMyD88

Myc-PKCε

Total Cell lysate
Figure 6:

A

B

C

Vector
WT C6
AA C11

Vector
WT C6
AA C11
WT Pool
AA Pool

GFP-PKCε
GFP-PKCε

LTA 20μg/ml

0 30 60 90

0 30 60 90

0 30 60 90

Min.

GFP-PKCε

IκBα

Tubulin
The scaffold MYD88 acts to couple protein kinase C epsilon (PKC<IMG SRC="/math/epsilon.gif" ALIGN="BASELINE" ALT="epsilon ">) to toll-like receptors
Amir Faisal, Adrian Saurin, Bernard Gregory, Brian Foxwell and Peter J. Parker

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