Protein kinase Cδ binds TIRAP/Mal to participate in TLR signaling

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Abbreviations: LPS, lipopolysaccharide; TLR, Toll-like receptor; CBB, coomassie brilliant blue; MAPK, MAP kinase; shRNA, short hairpin RNA

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Abstract

Toll-like receptor (TLR) family members recognize specific molecular patterns within pathogens. Signaling through TLRs results in a proximal event that involves direct binding of adaptor proteins to the receptors. We observed that TIRAP/Mal, an adaptor protein for TLR2 and TLR4, binds protein kinase Cδ (PKCδ). TIRAP/Mal GST-fusion protein and a TIRAP/Mal antibody were able to precipitate PKCδ from rat peritoneal macrophage and THP1 cell lysates. Truncation mutants of TIRAP/Mal showed that the TIR domain of TIRAP/Mal is responsible for binding. TLR2- and TLR4-mediated phosphorylation of p38 MAPK, IKK, and IκB in RAW264.7 cells were abolished by depletion of PKCδ. These results suggest that PKCδ binding to TIRAP/Mal promotes TLR signaling events.

Keywords; PKCδ; TLR; macrophage; signal transduction

1. Introduction

The Toll-like receptor (TLR) family plays a central role in regulating innate immunity. TLRs recognize conserved microbial products such as lipopolysaccharide (LPS), peptidoglycan, flagellin, and unmethylated CpG motifs in bacterial DNA (Kaisho and Akira, 2001). TLR signaling is initiated from the Toll-interleukin-1 receptor (TIR)-domain, a ~200 amino acid motif found in the cytosolic domain of all TLRs and the IL-1 receptor. Some adaptor molecules also contain TIR domains and bind to the TIR domains of TLR through stimulation-induced TIR/TIR interactions (Akira, 2003; Yamamoto et al., 2003). The TLR adaptor family has four known members, MyD88, TIRAP/Mal, TICAM-1/TRIF, and TICAM-2/TRAM. Studies with mice that lack the individual adaptors have defined a specific role for each one. MyD88 is shared by all TLRs except TLR3, while TIRAP/Mal only binds to TLR2 and TLR4, TICAM-1 binds exclusively to TLR3 and TLR4, and TICAM-2 only binds to TLR4 (Yamamoto et al., 2004; Oshiumi et al., 2003a; Oshiumi et al., 2003b). The diversity of adaptor molecules may partially account for the variety of inflammatory responses to different ligands.

MyD88 was the first adaptor protein to be identified, and its signaling pathway is now well characterized (Akira, 2003). Once MyD88 is recruited to the TLR, it associates with the serine/threonine kinases, IRAK-1 (IL-1R associated kinase-1) and IRAK-4. This association leads to IRAK-1 phosphorylation by IRAK-4 or other IRAK-1 molecules, a key event that allows IRAK-1 to bind TRAF6. This complex activates TAK-1, a MAPKKK family member, which activates SAPK/JNK and p38MAPK. TAK1 acts at a divergence point in the TLR signaling pathway because it can also activate IKK, a protein kinase responsible for IκB degradation and subsequent NF-κB activation.

The PKC family consists of at least 11 isoforms that are classified into three main subfamilies based on their homology and cofactor requirements for activation: the conventional PKCα, βI, βII and γ subfamily, which is diacylglycerol (DAG)- and calcium-dependent, the novel PKCδ, ε, θ and η subfamily, which is DAG-dependent and calcium-independent, and the atypical PKCζ, λ and ι family, which is phospholipid-dependent. There is growing evidence for a fundamental role of the PKC isoforms, PKCδ (Platten et al., 2003), ε (Aksoy et al., 2004; Castrillo et al., 2001), η (Chen et al., 1998), ι (Mamidipudi et al., 2004), and ζ (Hu et al., 2002; Monick et al., 2000), in the regulation of LPS-induced events in macrophages and dendritic cells. However, it is still unknown how PKCs are integrated into the TLR signaling pathway. In this study, we identified PKCδ as a signaling molecule that binds to TIRAP/Mal. Functional analysis suggests that PKCδ participates in the activation of both NF-κB and MAPK cascades induced by TLR signaling.

2. Materials and Methods

2. 1. Materials

LPS (E. coli, serotype 0111: B4), bovine serum albumin (fatty acid free), GF109203X, staurosporine, histone III-S, poly I:C, and ATP were purchased from Sigma (St. Louis, MO). Phosphatidylserine and phosphatidylinositol were bought from Matreya (Pleasant Gap, PA), raytide, a peptide substrate of tyrosine kinases, was purchased from Oncogene Science (Uniondale, NY), rGM-CSF came from PeproTech EC (London, UK);, Glutathione-Sepharose CL-4B and pGEX4T2 were bought from Amersham Biosciences (UK), antibodies raised against PKCs were bought from Transduction Laboratories (Lexington, KY), anti-phospho-p38 MAPK,

anti-phospho-SAPK/JNK, and anti-phospho-IKK were bought from Cell Signaling (Beverly, MA), and anti-phospho-IκB came from Santa Cruz (Santa Cruz, CA). Anti-TIRAP/Mal and anti-IRAK polyclonal antibodies were prepared by immunizing rabbits with full-length rTIRAP/Mal or recombinant C-terminal IRAK-1 peptide (636-end), respectively. [γ-^{32}P]ATP was purchased from New England Nuclear, and Phos-tag (1,3-bis[bis(pyridine-2-ylmethyl)amino] propan-2-olato dizinc(II) complex (Kinoshita et al., 2006) was kindly donated by Dr. T. Koike (Hiroshima University).

2. 2. Plasmids

Total RNA was extracted from human monocytes that had been cultured for ten days with 10 ng/ml GM-CSF using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Complementary DNA from TIRAP/Mal, IRAK-1, and MyD88 were amplified by RT-PCR using primers based on the sequences of the GenBank accession numbers AF406652, L76191 and U70451, respectively. The coding sequences for TIRAP/Mal and MyD88 were subcloned into pGEX-4T-2 in-frame with the GST coding sequence. TIRAP/Mal was also cloned into pcDNA3.1 with an N-terminal HA-tag. pcDNA3-FLAG-PKCδ (GenBank accession number M18330) was kindly donated by Dr. Yoshitaka Ono (Biomedical Research Center, Kobe). Truncation mutants of HA-Mal were made by PCR.

2. 3. Cells

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui, Japan) supplemented with 10% FCS in humidified 5% CO_2 at 37°C. A mouse macrophage-like cell line, RAW264.7, was maintained in RPMI1640 medium fortified with 2.5g/L glucose and 10% FCS in humidified 5% CO_2 at 37°C. Peritoneal macrophages were harvested from female Donryu rats. Briefly, animals were injected intra-peritoneally with 20 ml of a 3% thioglycollate broth. After four days, peritoneal exudate cells were harvested by washing the peritoneal cavity with ice-cold PBS. The cells were resuspended in RPMI1640 medium supplemented with 10% FCS and seeded at approximately $1.5x10^7$ cells/10-cm dish for the pull-down assay, or $3x10^6$ cells/well of a 6-well plate for immunoprecipitation, and incubated in humidified 5% CO_2 at 37°C for 1-2 h. Non-adherent cells were removed by washing vigorously with PBS and the attached cells were

used as macrophages. Macrophages were incubated in humidified 5% CO_2 at 37°C in the presence or absence of LPS.

2. 4. Transfection

293T cells were cultured in 6-well plates prior to the protein-protein interaction assay. The cells were seeded at $5x10^5$ cells/well for 24h before transfection using FuGENE 6 (Roche) according to the manufacturer's protocol. After 24 h, cells were lysed for immunoprecipitation. To detect expression of IRAKs and PKCs, 293T cells were plated at $5x10^5$ cells/10-cm dish for 24 h before calcium phosphate transfection. The media was changed 20-24 h after transfection, and 48 h later the cells were lysed for immunoprecipitation.

2. 5. GST-fusion proteins

BL21 (DE3) cells were transformed with the GST-TIRAP/Mal or GST-MyD88 plasmid, and grown to mid-log phase. Protein production was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 30°C. The cells were harvested by centrifugation, lysed with lysis buffer (TBS containing 0.5% Triton X-100 and 1 mM PMSF), and sonicated 3x for 30 sec with a needle sonicator at 40-50% power. The clarified supernatants were incubated with glutathione-Sepharose at 4°C for 60 min. The beads containing the recombinant proteins were washed six times with lysis buffer.

2. 6. Pull-down with GST-proteins

After removal of cell culture supernatants, the cells were washed once with ice-cold PBS containing 1 mM EDTA, 10 mM NaF and 1 mM Na_3VO_4, and immediately incubated in 1 ml of lysis buffer (1 mM EDTA, 25 mM Tris-HCl (pH7.4), 100 mM NaCl, 30 mM NaF, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM PMSF, 2 μM leupeptin, and 20 μM PA-PMSF) for 10 min on ice. The cell lysates were centrifuged at 15,000 rpm for 10 min. The resultant supernatants were precleared with glutathione-Sepharose at 4°C for 60 min and incubated with the GST-fusion proteins immobilized on glutathione-Sepharose at 4°C for 120 min. The Sepharose beads with the fusion proteins were spun briefly in a microfuge and washed four times with ice-cold lysis buffer. For the *in*

vitro kinase assay, the beads were further washed with kinase assay buffer (25 mM Tris-HCl, pH7.5, 0.1 mM EDTA, and 0.1 mg/ml BSA).

2. 7. Immunoprecipitation

Cell lysates were prepared as above, and incubated with the appropriate antiserum for at least 60 min on ice. A slurry of pre-washed protein A-Sepharose CL-4B (30% slurry, 50 μl) was added to each sample and incubated on a rotator at 4°C for an additional 2 h. The samples were spun briefly in a microfuge and washed four times with ice-cold lysis buffer. For the *in vitro* kinase assay, the beads were further washed with kinase assay buffer as described above.

2. 8. In vitro kinase assay

The kinase activities of the GST-TIRAP/Mal and GST-MyD88 fusion proteins were determined by incubating the glutathione-beads with [γ-^{32}P]ATP and protein substrates. The glutathione-beads were suspended in 20 μl kinase assay buffer containing the appropriate substrates and protein kinase inhibitors. Unless otherwise specified, phospholipids were added to the reaction mixture as lipid vehicles made by sonication. The reaction was initated by adding 10 μl of the reaction mixture fortified with 0.3 mM [γ-^{32}P]ATP (1 μCi), 5μg histone III-S and 30 mM MgCl2, continued at 30˚C for 60 min, and terminated by addition of SDS sample buffer. After separation of the proteins on SDS-PAGE, the gels were stained with coomassie brilliant blue (CBB) and the protein bands were analyzed with an imaging analyzer to estimate the degree of substrate phosphorylation. Anti-IRAK and anti-flag-PKC immunoprecipitates were also assessed using the methodology described above.

2. 9. Western blotting

GST-fusion proteins immobilized on glutathione-Sepharose beads, anti-TIRAP/Mal immunoprecipitates, anti-flag immunoprecipitates of PKCδ, or total cell lysates were heated at 100˚C for 3 min in 30 μl of the

sample buffer containing 62.5 mM Tris (pH 6.8), 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.02% bromophenol blue. The proteins were separated by SDS-PAGE on a 10% slab gel and transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were blocked, incubated with the appropriate antibodies, and visualized by enhanced chemiluminescence.

2. 10. RNA Interference

Two sets of oligonucleotides were cloned into the pH1 vector to express PKCδ siRNA hairpins downstream of the human H1 RNA promoter, as described previously (Sasai et al., 2005). The following sequences were used: 5'-GAACGCTTCAACATCGACA-3 and 5'-GGCCGTGTTATCCAGATTG-3. 293 cells were transfected with 100 ng of pH1-empty or 50 ng of each pH1-siPKCδ vector using FuGENE6 Transfection Reagent. 5-10x10^6 RAW264.7 cells were transfected by electroporation in a 300 μL final volume at 250 V/950μF (Gene Pulser II, Bio-Rad). 24 h after transfection, puromycin (2 μg/mL for 293T cells and 5 μg/mL for RAW264.7 cells) was added to the cells, and incubation was continued for several days. Puromycin-resistant colonies were replated. To determine the efficiency of gene silencing, total RNA was isolated with RNeasy (Qiagen, Hilden, Germany) and mRNA was assessed by RT-PCR.

3. Results

3. 1. GST-TIRAP/Mal fusion protein associates with protein kinases

We first determined whether TIRAP/Mal interacts with protein kinases. Recombinant GST-TIRAP/Mal was incubated with rat peritoneal macrophage lysates. Proteins associating with the fusion protein were analyzed in the *in vitro* kinase assay using histone III-S as a substrate. Phosphorylation of histone III-S was prominent when phospholipids were included in the assay mixture (Fig. 1, upper panel). TIRAP/Mal and histone III-S levels remained unchanged during the kinase assay (Fig. 1, lower panel), and GST alone was not associated with protein kinases activity (Fig. 1B). A tyrosine kinase substrate, raytide, was not phosphorylated by TIRAP/Mal-associated kinase (data not shown). Since PKC family members are phospholipid-dependent, we

tested the effect of PKC inhibitors on the protein kinase activities. A PKC inhibitor with a broad spectrum, GF109203X, inhibited histone III-S phosphorylation by TIRAP/Mal-associated kinases, while Gö6976, a specific inhibitor of conventional PKCs, did not (Fig. 2A, upper panels). MyD88 also associated with some protein kinases, as shown by histone III-S phosphorylation (Fig. 2B). MyD88-associated activity was susceptible to both GF109203X and Gö6976 (Fig. 2B, upper panels) indicating that the bound kinases are different from those associated with TIRAP/Mal. Another PKC inhibitor, staurosporine, strongly inhibited both MyD88-associated and TIRAP/Mal-associated kinases (data not shown). Since MyD88 is known to bind IRAK family members, we analyzed these as potential TIRAP/Mal-associated protein kinases. However, GF109203X, which inhibited protein kinase activity associated with TIRAP/Mal (Fig. 2A, left upper panel), failed to inhibit kinase activity in lysates from IRAK-1 and IRAK-4 transfected 293T cells (data not shown).

3. 2. Identification of PKCδ and η as TIRAP/Mal-associated kinases

Whole cell lysates of rat macrophages were western blotted with a series of specific antibodies against the PKC isoforms. As shown in Fig. 3A, PKCδ, ι, and λ were the dominant PKC subtypes observed by the employed antibodies, though PKCα and β were also observed. The lysates were mixed with GST-TIRAP/Mal immobilized on glutathione-Sepharose beads, and protein associated with the beads was western blotted with specific antibodies. PKCδ and η bound GST-TIRAP/Mal (Fig. 3A), while GST alone did not bind any PKCs (data not shown). A point mutation of TIRAP/Mal (P125H) known to impair the ability of this adaptor to bind TLR4 and MyD88 (Horng et al., 2001; Fitzgerald et al., 2001; Xu et al., 2000), did not alter the ability of TIRAP/Mal to bind PKCδ and η (Fig. 3A). PKCδ and η binding to TIRAP/Mal was also observed in the lysates of monocytic THP-1 cells (data not shown).

To confirm the binding of TIRAP/Mal to PKCδ, RAW264.7 cells were transfected with shRNA probes that targeted PKCδ. The probes prevented PKCδ expression without affecting expression of a control protein, $p67^{phox}$ (see Fig. 5). Lysates of PKCδ-depleted cells were incubated with recombinant GST-TIRAP/Mal and protein associated with this fusion protein was assessed using the *in vitro* kinase assay. Depletion of PKC δ markedly decreased the protein kinase activity associated with GST-TIRAP/Mal (Fig. 1B). As shown in Fig. 3B,

a polyclonal antibody against TIRAP/Mal was used to analyze its interaction with PKC δ. This antibody was incubated with rat peritoneal macrophage lysates, and the immune complex was analyzed using a monoclonal antibody against PKCδ. The results confirmed that TIRAP/Mal associates with PKCδ and showed that this association is constitutive, remaining unchanged after treatment with LPS. The anti-TIRAP/Mal antibody precipitated the histone III-S kinase activity from wild type RAW264.7 cell lysates (Fig. 3C), and kinase activity also remained unchanged by LPS treatment. When a similar experiment was performed using the lysate from PKCδ-deficient cells, kinase activity was barely detected in the immune complex (Fig. 3C). As shown in Fig. 3D, RAW264.7 cell lysates were mixed with anti-PKCδ, and the immune complex was analyzed by Phos-tag, a probe used to detect phosphorylated proteins (Kinoshita et al., 2006). The proteins in the immune complex of anti-PKCδ were separated by SDS-PAGE, transferred to PVDF membrane, and analyzed by Phos-tag. Interestingly, LPS increased the phosphorylation of several proteins in the immune complex. PKCδ was constitutively phosphorylated, however, the level of phosphorylation was unaffected by LPS treatment.

293T cells were then transfected with flag-PKCδ along with HA-TIRAP/Mal (Fig. 4). The cell lysate was mixed with an anti-flag antibody, and immune complexes were analyzed using an anti-HA antibody. The results confirmed that PKCδ binds to the full length TIRAP/Mal (WT). The TIR domain of TIRAP/Mal (86 to end) bound flag-PKCδ, while HA-TIRAP/Mal lacking the TIR domain (ΔTIR) did not complex with flag-PKCδ.

3. 3. Possible involvement of PKCδ in LPS-induced activation of MAPKs and IKK

TLR ligation is known to activate MAPKs and IKK. As shown in Fig. 5A, stimulation of RAW264.7 cells with LPS, a TLR4 ligand, induced the phosphorylation of IKK, p38 MAPK, and IκB. The LPS-induced events were completely abolished in PKCδ-deleted cells (Fig. 5A). Similar results were obtained in cells treated with Malp2, a TLR2/TLR6 ligand (Fig. 5B). These results indicate that PKCδ plays a critical role in TIRAP/Mal-dependent signaling pathways. Poly I:C is a TLR3 ligand that binds TICAM-1 but not TIRAP/Mal. Poly I:C, like LPS and Malp2, induced the phosphorylation of IKK, p38 MAPK, and IκB (Fig. 5C). In PKCδ-deficient cells, IKK and IκB phosphorylation were severely impaired but still observable (Fig. 5C). Interestingly, the phosphorylation of p38 MAPK was not altered by the 60-90 min treatment (Fig. 5C).

Phosphorylation induced by calyculin A, a pan-inhibitor of Ser/Thr phosphatases, was not affected by PKCδ-depletion (Fig. 5D).

4. Discussion

Numerous studies have reported that LPS induces PKC activation (Aksoy et al., 2004; Shinji et al., 1994). A deficiency in LPS-induced translocation of novel and conventional PKC is observed in macrophages from C3H/HeJ mice, which harbor a TLR4 point mutation (Shinji et al., 1994). PKCζ, an atypical PKC subfamily member, is proposed to function in LPS-induced IRAK activation (Hu et al., 2002), and PKCε, a novel PKC, is indispensable for LPS-induced IL-12 production by dendritic cells (Aksoy et al., 2002). PKCε-deficient murine macrophages display a major defect in their capacity to clear bacterial infection (Castrillo et al., 2001).

It is reported that LPS-induced activation of NF-κB is inhibited by rottlerin (Kontny et al., 2000). Although rottlerin is often utilized as an inhibitor of PKCδ, a recent study indicated that the compound do not inhibit PKCδ (Davies et al., 2000). We confirmed the inability of rottlerin to inhibit PKCδ using a recombinant enzyme (data not shown). Thus, the present study was performed to provide a new line of evidence suggesting that PKCδ is involved in TLR signaling. We observed that the adaptor protein, TIRAP/Mal, is associated with protein kinase activity (Fig. 2) that is unaffected by rottlerin (data not shown). The effects of phospholipid and pharmacological inhibitors suggested involvement of PKC family members. Immunological analysis indicated that PKCδ and η, members of the novel PKC subfamily, were associating with TIRAP/Mal (Fig. 3A). Indeed, truncated mutants of TIRAP/Mal showed that the TIR domain is required for binding to PKCδ (Fig. 4). PKCε, another novel isoform, is recently shown to be a critical component of the TLR4 signaling pathway (Aksoy et al., 2002). It appears that some structures that are common in novel PKC family members may associate favorably with TIRAP/Mal.

Constitutively active PKCθ, another novel PKC isoform, is a potent activator of NF-κB and AP-1 in Jurkat T cells (Lin et al., 2000; Baier-Bitterlich et al., 1996). Activation of IKK and NF-κB by TCR signaling depends on PKCβ or novel PKCs (Shinohara et al., 2005; Sommer et al., 2005; Krappmann et al., 2001). Although this finding suggests that novel PKCs are involved in the signaling pathway leading to NF-κB

activation, it is not clear whether each PKC isozyme has a specialized function. The substrate specificity of PKC isoforms is redundant, so their physiological roles in certain cell types may depend on their relative abundance, localization, and cellular environment. In this study, we observed that TLR2- and TLR4-mediated activation of IKK was completely abolished in PKCδ-deficient RAW264.7 cells (Fig. 5). Considering that PKCδ is the most abundant novel PKC in macrophages, it is not surprising that this subtype plays a dominant role in these cells. In fact, activation of NF-κB by TLR2, TLR4, TLR3, and TLR9 was also attenuated in PKCδ-deficient cells (data not shown). TLR3-mediated responses were attenuated but still observed in these cells, while the TLR2/4 responses were completely abolished (Fig. 5). Therefore, we have to consider two independent roles of PKCδ in TLR signaling. One is specific for TLR2/4, and another is common to all TLRs.

TICAM-1/TRIF did not bind to PKCδ (data not shown). In addition, ligation of TLR3 with polyI:C did not increase the phosphorylation of PKCδ-associated proteins (data not shown) where TLR4 ligation considerably increase the phosphorylation (Fig. 3D). It is very likely that PKCδ interacts specifically with TIRAP/Mal. There may be another mechanism by which PKCδ regulates TLR-mediated signals. We have recently observed that phosphatidylinositol 3-kinase (PI3K) and Akt activity is greatly increased in the PKCδ deficient cells (to be published elsewhere). As reported by numerous studies and also by us, activation of PI3K/Akt resulted in a suppression of TLR-mediated NF-κB activation. The negative regulation by PI3K of TLR is commonly observed with all TLR signals (Hazeki et al., 2006). Based on this, the augmentation of PI3K activity in PKCδ deficient cells may be responsible for the general impairment of TLR signals.

TIRAP/Mal is only shared by TLR2 and TLR4, while MyD88 is shared by all the TLRs (Yamamoto et al., 2003). Thus, TIRAP/Mal-binding proteins are good candidates for a signaling molecule that induces specific responses to TLR2 and TLR4. Although the signaling pathway downstream of MyD88 is well characterized, little is known about TIRAP/Mal signaling except that it specifically associates with PKR (Horng et al., 2001) and IRAK-2 (Fitzgerald et al., 2001). This study suggests that PKCδ also interacts specifically with TIRAP/Mal. Interactions between TIRAP/Mal and Btk, a Tec family tyrosine kinase, are also reported. TIRAP/Mal is phosphorylated by Btk and subject to proteosomal degradation, which results in negative feedback termination of the innate immune response (Mansell et al., 2006). Interestingly, PKCδ inhibits the function of Tec family tyrosine kinases (Saharinen et al., 1997). It is intriguing to consider that the inhibition of Btk is one of the

specialized functions of PKCδ. Another recent study revealed that TIRAP/Mal associates with TRAF6 (Mansell et al., 2004), which binds to numerous functional proteins including MyD88 (Kawai et al., 2004), IRAK-1 (Jiang et al., 2002), TRIF (TICAM-1; Sato et al., 2003), TBK-1 (Sato et al., 2003), TAB1/2 (Qian et al., 2001), TAK-1 (Qian et al., 2001), IRF-7 (Kawai et al., 2004), ECSIT (Kopp et al., 1999), β-arrestin (Wang et al., 2006), Src family tyrosine kinase (Wong et al., 1999), Ras (McDermott and O'Neill, 2002), and aPKC-interacting p62 (Sanz et al., 2000). These proteins are candidates for regulation by PKCδ, as described below.

We showed that LPS increases the association between phosphorylated proteins and PKCδ (Fig. 3D). PKCδ is activated when tyrosine residues on this protein become phosphorylated (Konishi et al., 2001). Since ligation of TLR4 increases tyrosine kinase activity in cells (Hazeki et al., 2003), it is possible that tyrosine-phosphorylated PKCδ phosphorylates other proteins. We did not observe any effect of LPS on the tyrosine-phosphorylation of PKCδ, however (data not shown), and the PKCδ activity associated with TIRAP/Mal was also unaffected by LPS (Fig. 3C). Another possibility is that PKCδ is recruited to the TLR signaling complex, since PKC translocation is often observed in intact cells. We have observed that the association between PKCδ and TIRAP/Mal is not dependent on receptor ligation (Fig. 3B). Thus, LPS-induced recruitment of TIRAP/Mal to the TLR or TRAF6 may be the event that results in increased phosphorylation of proteins in the immune complex formed by anti-PKCδ. LPS induces the association between TIRAP/Mal and TRAF6, which binds to PKCδ and its substrates, respectively. Further study is necessary to define the detailed signaling mechanism.

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FIGURE LEGENDS

Fig. 1. Association of protein kinase activity with GST-TIRAP/Mal. (A) GST-TIRAP/Mal immobilized on glutathione-Sepharose beads were incubated with rat macrophage lysates. The beads were washed four times and assayed for protein kinase activity using [γ-^{32}P]ATP and histone III-S as substrates in the presence (+) or absence (-) of phosphatidylserine and diacylglycerol (PS/DG). The reaction was terminated by the addition of SDS sample buffer. (B) GST-TIRAP/Mal or control GST immobilized on glutathione-Sepharose were incubated with lysates from wild type RAW264.7 cells or PKCδ deficient cells ($\Delta\delta$). Protein kinase activity associated with the beads was determined in the presence of the phospholipids. (A, B) The phosphorylated proteins were visualized using an imaging analyzer (Autoradiography). Histone III-S and the fusion proteins were stained with CBB on the same gels. Positions of molecular mass markers (kDa) are indicated on the left of the panel, while the positions of the proteins are indicated on the right in (A). The position of the proteins are indicated on the left of the panel in (B).

Fig. 2. Effects of PKC inhibitors on the protein kinase activity bound to GST-TIRAP/Mal and GST-MyD88. GST-TIRAP/Mal (A) or GST-MyD88 (B) immobilized on glutathione-Sepharose was incubated with rat macrophage lysates. The beads were washed and assayed for protein kinase activity using [γ-^{32}P]ATP and histone III-S. Increasing concentrations of GF109203X or Gö6976 were added to the assay mixture. Protein phosphorylation was visualized with an imaging analyzer (upper panels). The amounts of histone III-S and fusion proteins on the same gels were detected by CBB staining (lower panels). Positions of the molecular mass markers (kDa) are indicated on the left of the panels, while the positions of the proteins are indicated on the right.

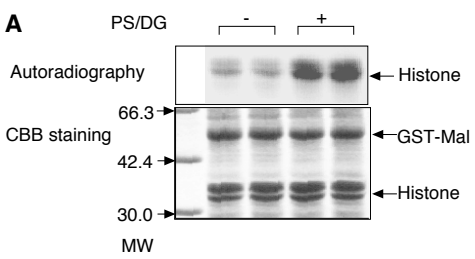
Fig. 3. PKCδ binds GST-TIRAP/Mal. (A) GST-TIRAP/Mal or its mutant (P125H) immobilized on glutathione-Sepharose were incubated with rat macrophage lysates. The bound proteins were analyzed by immunoblotting with a series of anti-PKC antibodies as indicated at the top of the panel. Total macrophage lysate was also immunoblotted (top panel). (B) Rat macrophages were stimulated with 100 ng/ml LPS for the

times indicated at the top of the panel. The cell lysates were immunoprecipitated with anti-TIRAP/Mal antibody or control IgG and the proteins in the immune complex were analyzed by immunoblotting with anti-PKCδ. Total cell lysates were also analyzed as a positive control. The positions of PKCδ and TIRAP/Mal are indicated on the left of the panel. (C) Wild type RAW264.7 cells or PKCδ-dedficient cells (Δδ) were stimulated with LPS. The lysates were immunoprecipitated with anti-Mal antibody and the immunoprecipitates were analyzed by *in vitro* kinase assay. (D) Wild type RAW264.7 cells were stimulated with 100 ng/ml LPS. The cell lysates were immunoprecipitated with anti-PKCδ antiboody and phosphorylated proteins were detected using Phos-tag.

Fig. 4. The TIR domain of TIRAP/Mal associates with PKCδ. 293T cells were transfected with 0.5 μg of flag-PKCδ with HA-TIRAP/Mal (WT), HA- TIRAP/Mal-TIR (TIR, 86-end), or HA-TIRAP/Mal-ΔTIR (ΔTIR, 1-85) as indicated. PKCδ was immunoprecipitated from the cell lysates with an anti-flag antibody. The proteins in the immune complex were separated by SDS-PAGE and blotted with an anti-HA antibody (top panel) or anti-flag antibody (middle panel). The amount of HA-TIRAP/Mal in the total cell lysate was determined by blotting with an anti-HA antibody (lower panel). The positions of the proteins are indicated on the left of the panel.

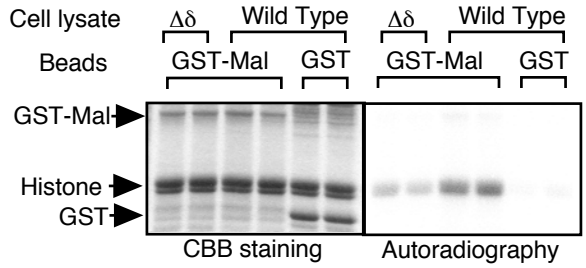
Fig. 5. Possible involvement of PKCδ in TLR-mediated activation of p38MAPK and NF-κB. RAW264.7 cells were transfected with two PKCδ-shRNAs (10 μg each) or empty vector (20 μg) by electropotation. After 24 h, 7 μg/mL of puromycin was added and the cells were cultured for four weeks, changing the medium every five days. The resistant colonies were plated in fresh medium without puromycin and cultured for an additional 24 h. The cells were stimulated with 10 ng/ml of LPS (A), 0.1 μM of Malp2 (B), or 25 μg/mL of Poly I:C (C) for the times indicated at the top of the panels. In (D), the cells were treated with 0.1 μM calyculin A for 15 min. The cell lysates were western blotted with the antibodies indicated at the left of the panels.

Figure

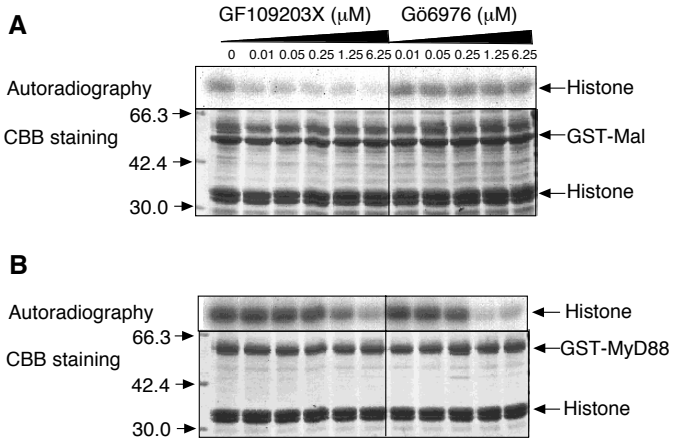


Murai, M. et al. Fig. 1A

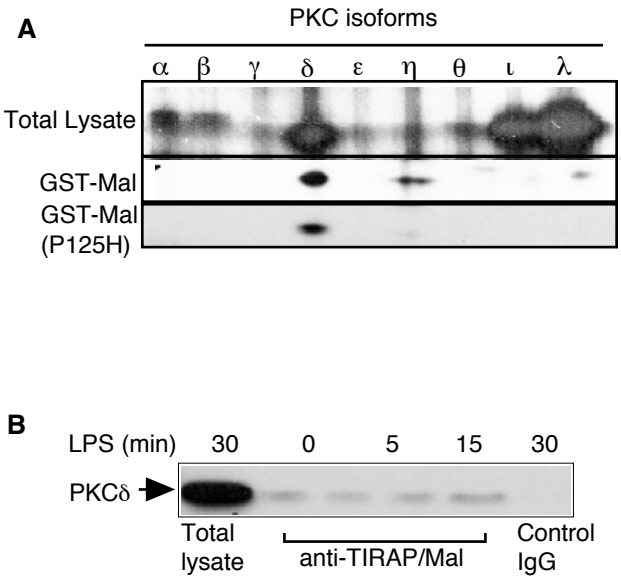
B



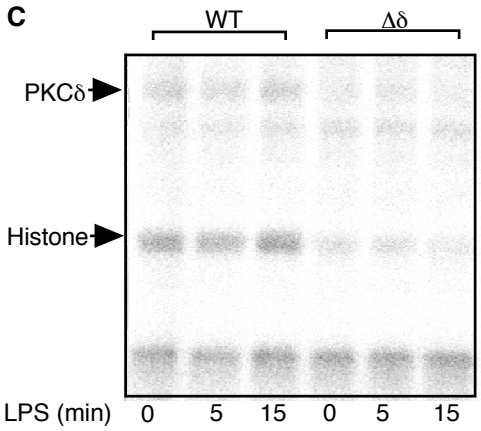
Murai, M. *et al.* Fig. 1B

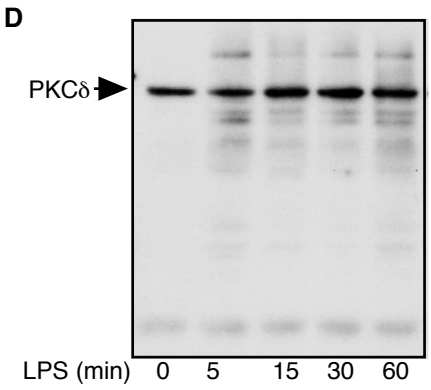


Murai, M. et al. Fig. 2

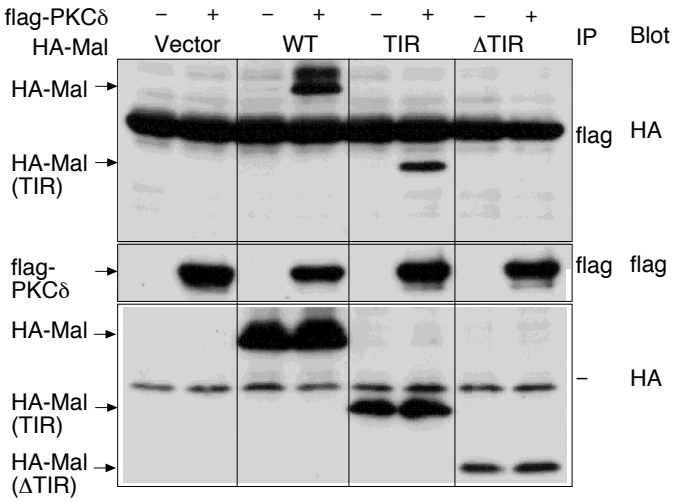


Murai, M. *et al.* Fig. 3 (A, B)

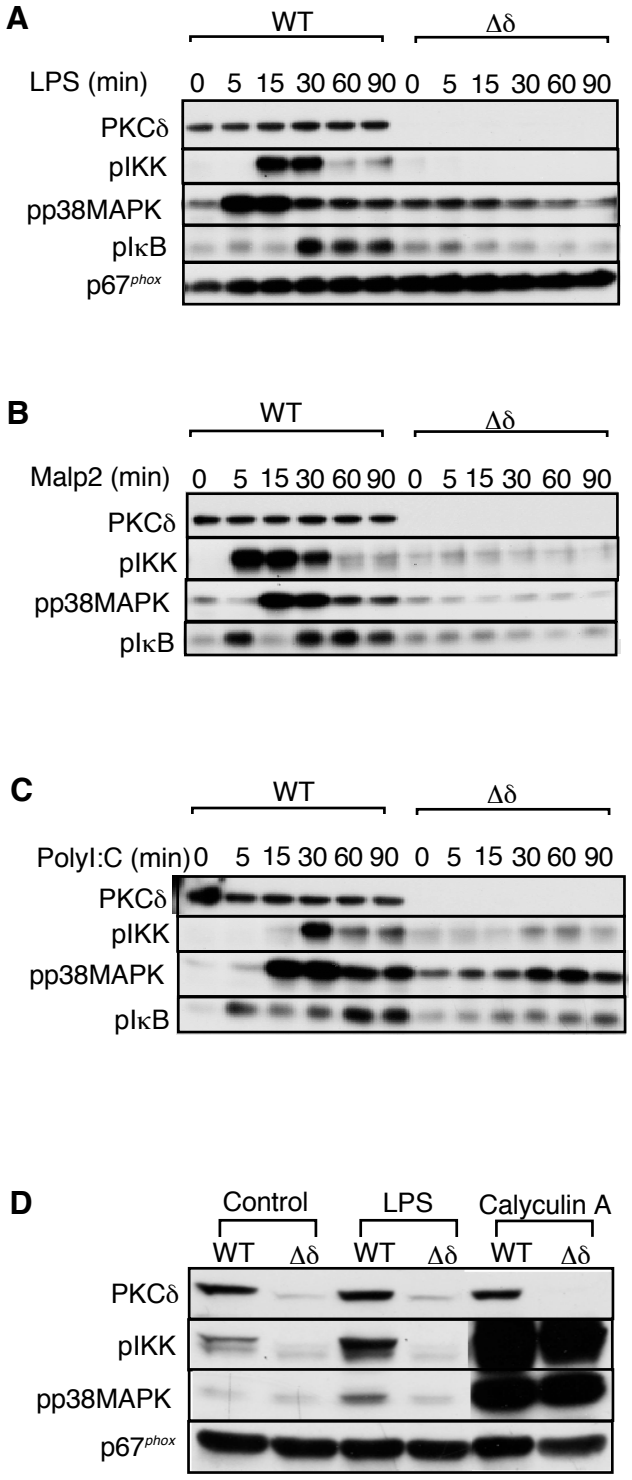




Murai, M. *et al.* Fig. 3 (C, D)



Murai, M. et al. Fig. 4



Murai, M. *et al.* Fig. 5