Trif-related adapter molecule is phosphorylated by PKCε during Toll-like receptor 4 signaling

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PKCε has been shown to play a key role in the effect of the Gram-negative bacterial product LPS; however, the target for PKCε in LPS signaling is unknown. LPS signaling is mediated by Toll-like receptor 4, which uses four adapter proteins, MyD88, MyD88 adapter-like (Mal), Toll/IL-1R domain-containing adapter inducing IFN-β (Trif), and Trif-related adapter molecule (TRAM). Here we show that TRAM is transiently phosphorylated by PKCε on serine-16 in an LPS-dependent manner. Activation of IFN regulatory factor 3 and induction of the chemokine RANTES, which are both TRAM-dependent, were attenuated in PKCε-deficient cells. TRAMS16A is inactive when overexpressed and is attenuated in its ability to reconstitute signaling in TRAM-deficient cells. We have therefore uncovered a key process in Toll-like receptor 4 signaling, identifying TRAM as the target for PKCε.

innate immunity | LPS | myristoylation

The PKC family of serine/threonine kinases play a critical role in the immune response (1). The family consists of at least 11 members divided into three subgroups (2). The conventional α, βI, βII, and γ PKC isotypes are diacylglycerol (DAG)-dependent and calcium-dependent; the novel δ, ε, θ, and η PKC isotypes are DAG-dependent but calcium-independent; and the atypical ξ and λ/ι PKC are DAG-independent. Several PKC isoenzymes have been implicated in the immune response. Mice deficient in PKC βI and βII display an impairment in signaling through the B cell receptor (3), whereas mice deficient in PKCθ have a defect in T cell receptor signaling, resulting in a severe decrease in NF-κB activation and mature T cell proliferation (4). Inhibition of PKCζ causes an attenuation in the activation of Toll-like receptor (TLR) 4-signaling components upon LPS stimulation in macrophages (5), whereas Dallot *et al.* (6) demonstrated a role for PKCζ in LPS-induced nuclear translocation of the p65 subunit of NF-κB in human myometrial cells. PKC$\varepsilon^{-/-}$ mice appear normal and are generally healthy, but they display a major defect in clearing bacterial infections (7). Effects of the Gram-negative bacterial product LPS are particularly impaired in PKC$\varepsilon^{-/-}$ mice. LPS-treated macrophages from PKC$\varepsilon^{-/-}$ mice showed reduced expression of nitric oxide, TNF-α, and IL-1β and reduced activation of NF-κB and the mitogen-activated protein kinases p42/p44 and p38; however, the target for PKCε in LPS signaling is unknown.

LPS signaling is mediated by TLR4 (8, 9). TLR4 signaling involves four adapter proteins, MyD88, MyD88-adapter like [Mal; also known as Toll/IL-1 receptor (TIR) domain-containing adapter molecule], TIR-containing adapter inducing IFN-β (Trif; also known as TIR-containing adapter molecule 1), and Trif-related adapter molecule (TRAM; also known as TIR-containing adapter molecule 2) (10). MyD88 and Mal work together to mediate early activation of NF-κB and mitogen-activated protein kinases, which leads to induction of cytokines such as TNF and IL1 (11), whereas Trif and TRAM activate another transcription factor, IFN regulatory factor 3 (IRF3), leading to the production of cytokines such as IFNβ and RANTES (12–14). IRF3 has also been shown to be involved in the induction of TNF, which in LPS signaling leads to late activation of NF-κB and mitogen-activated protein kinases (15, 16). The role of TRAM in LPS signaling appears to be to act as a bridging adapter connecting TLR4 and Trif.

The N terminus sequence of TRAM, MGIGKS, is a myristoylation consensus sequence (MGXXXS/T), and we recently found that TRAM is myristoylated (27). The myristate group facilitates membrane binding by simple insertion into the hydrophobic interior of the lipid bilayer and allows TRAM to colocalize with TLR4 in the plasma membrane and Golgi apparatus. Myristoylated proteins are often phosphorylated by PKC, leading to a dissociation from the membrane (17–19). This finding led us to examine whether TRAM might be an important substrate for PKCε in LPS signaling. Here we describe the phosphorylation of TRAM on serine-16 by PKCε and reveal that this process is an important regulatory mechanism in TLR4 signal transduction. We have therefore identified TRAM as a key target for PKCε in LPS signaling.

Results

TRAM Is Phosphorylated upon LPS Stimulation. We examined whether TRAM can undergo phosphorylation. Purified GST-TRAM was incubated with lysates from THP1 cells treated with various stimuli. Lysates from cells treated with LPS for 15 and 30 min phosphorylate TRAM (Fig. 1*a*). Lysates from cells treated with poly(I·C) were without effect (Fig. 1*b*). This effect was further confirmed in HEK293-TLR4 cells transfected with FLAG-TRAM and stimulated with LPS. FLAG-TRAM was immunoprecipitated from the cells, and lysates were blotted with an anti-phosphoserine antibody. TRAM showed an increase in serine phosphorylation in cells treated with LPS for 30 min (data not shown).

TRAM Is Phosphorylated by PKCε. The next step was to discover the kinase involved in the phosphorylation of TRAM. Several myristoylated proteins, including MARCKS and Src, are phosphorylated by PKC (17–20), so we wished to investigate whether TRAM was also phosphorylated by PKC. We incubated THP1 lysates from LPS-treated cells with various concentrations of the pan-PKC inhibitor bis(indolyl)maleimide (BIS). Increasing concentrations of BIS led to an inhibition in the ability of lysates to phosphorylate TRAM (Fig. 1*c*). Because PKCε and PKCζ are known to be involved in the LPS pathway (5–7, 21, 22), we next tested whether PKCε and/or PKCζ were phosphorylating TRAM. We first demonstrated that recombinant PKCε could phosphorylate TRAM. As shown in Fig. 1*d*, recombinant PKCε phosphorylated TRAM (lane 6), with no effect being evident on another adapter, Mal (lane 7). PKCε also underwent autophosphorylation (top band). PKCζ also underwent autophosphorylation (top band) but could not phosphorylate TRAM (lane 4). We next examined THP1 lysates, which

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Abbreviations: TIR, Toll/IL-1 receptor; Trif, Toll/IL-1R domain-containing adapter inducing IFN-β; TRAM, Trif-related adapter molecule; TLR, Toll-like receptor; MEF, murine embryonic fibroblast; BIS, bis(indolyl)maleimide; IRF3, IFN regulatory factor 3; DAG, diacylglycerol; Mal, MyD88 adapter-like.

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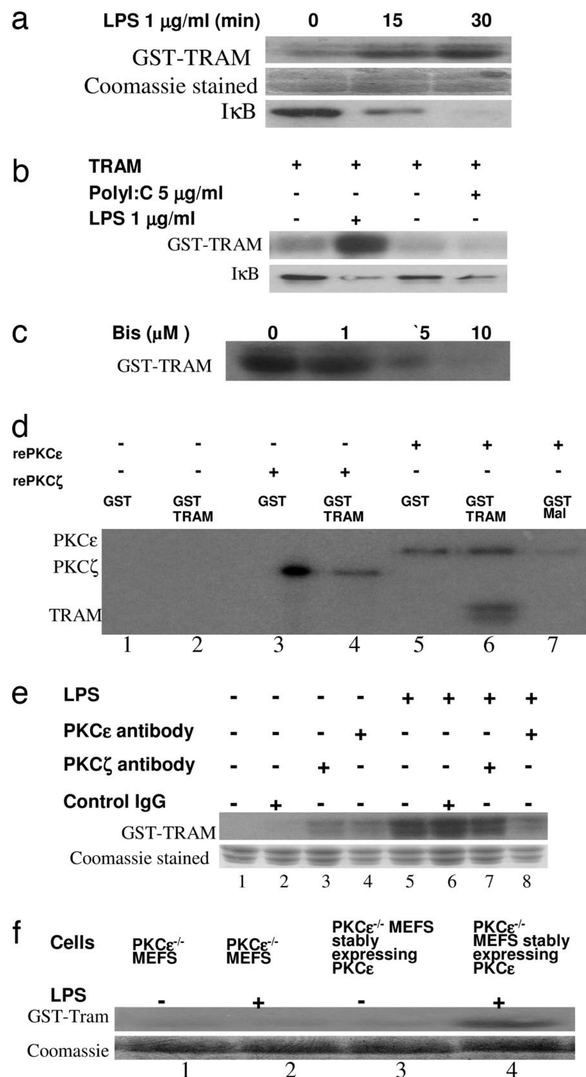

Fig. 1. TRAM is phosphorylated upon stimulation by LPS but not poly(I·C). (*a*–*c*) A kinase assay was performed on purified GST-TRAM and lysates from THP1 cells as described in *Methods*. (*b*) Lysates from THP1 cells stimulated with either LPS or poly(I·C) for 30 min. (*c*) Lysates from THP1 cells stimulated with LPS for 30 min were preincubated with increasing concentrations of the PKC inhibitor BIS for 1 h before incubation with GST-TRAM. (*d*) A recombinant *in vitro* kinase assay was performed with recombinant PKCε or PKCζ incubated with GST-TRAM, GST-Mal, or GST alone. (e) THP1 lysates were immunodepleted by using a PKCε-specific or PKCζ-specific antibody or an IgG control before a kinase assay was performed. (*f*) A kinase assay was performed on lysates from PKCε$^{-/-}$ MEFs and PKCε$^{-/-}$ MEFs that had been reconstituted with PKCε that had been stimulated with LPS for 30 min. Results shown are representative of at least three experiments.

were depleted of PKCε and PKCζ by using a PKCε- or PKCζ-specific antibody, respectively. The removal of PKCε abolished the ability of lysates from LPS-treated cells to phosphorylate TRAM (Fig. 1*e*, compare lane 8 with lane 6), whereas the removal of PKCζ had no effect (Fig. 1*e*, compare lane 7 with lane 6). To confirm PKCε involvement, lysates from PKCε$^{-/-}$ murine embryonic fibroblasts (MEFs) were next tested. As shown in Fig. 1*f*, lysates from PKCε$^{-/-}$ MEFs reconstituted with PKCε treated with LPS phosphorylate TRAM (lane 4), with no effect being evident in PKCε$^{-/-}$ MEFs (lane 2). Taken together these results identify TRAM as a substrate for PKCε.

Some myristoylated proteins, e.g., Src, are phosphorylated near their N termini (23, 24). We therefore next examined whether any of the serines close to the N-terminal myristoylation site were the

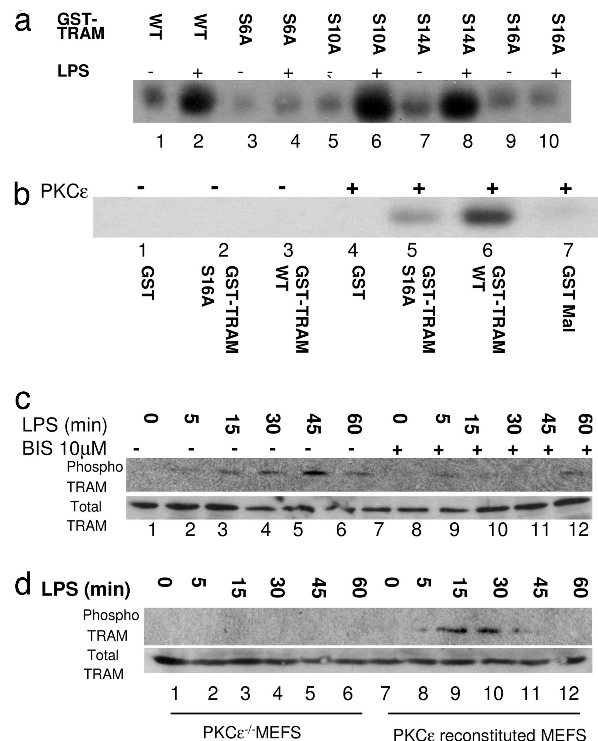

Fig. 2. TRAM is phosphorylated on serine-16 in an LPS-dependent manner. The first four serines at positions 6, 10, 14, and 16 were individually mutated to alanines (S6A, S10A, S14A, and S16A) and expressed and purified as GST fusions. A kinase assay was performed by using lysates from THP1 cells that had been stimulated with 1 µg/ml LPS for 30 min and the appropriate GST fusion (*a*) and recombinant PKCε and GST, GST-TRAMS16A, GST-TRAM, or GST-Mal (*b*). Endogenous TRAM phosphorylation on serine-16 was measured in lysates taken from THP1 cells incubated with and without the PKC inhibitor BIS for 1 h before stimulation with 1 µg/ml LPS (*c*) and PKCε$^{-/-}$ MEFs and PKCε$^{-/-}$ MEFs that had been reconstituted with PKCε after stimulation with 1 µg/ml LPS for the indicated lengths of time (*d*). Results shown are representative of at least three experiments.

target of PKCε. The first four serines in TRAM were individually mutated to alanines by using site-directed mutagenesis. Kinase assays were carried out by using GST-tagged versions of the mutants and lysates from LPS-treated THP1 cells. The phosphorylation of TRAM in response to LPS was completely abolished when serine-6 (Fig. 2*a*, lane 4) or serine-16 (lane 10) was mutated individually. Mutation of serine-10 or serine-14 had no effect on TRAM phosphorylation (lanes 6 and 8), suggesting that these serines are not involved in LPS-induced phosphorylation of TRAM. It is therefore likely that TRAM is phosphorylated on serine-6 and serine-16. Further analysis of serine-6 as a phosphoacceptor was not carried out because it was not possible to carry out functional studies on this mutant. Serine-6 occurs in the myristoylation consensus sequence, and we have found that mutating the myristoylation consensus sequence prevents TRAM from functioning correctly (27). However, our analysis of serine-16 revealed that phosphorylation of GST-TRAMS16A by recombinant PKCε was much lower than WT TRAM (Fig. 2*b*, compare lane 6 with lane 5). This result led us to test phosphorylation of endogenous TRAM on serine-16 by PKCε. We raised an antibody to a synthetic peptide comprising amino acids 7–21 of TRAM with a phosphoserine inserted instead of a serine at amino acid 16. Immunoblotting lysates from THP1 cells treated with LPS showed TRAM phosphorylation on serine-16 appearing after 15 min and peaking at 45 min (Fig. 2*c*, lanes 1–6). Incubation of these cells with the PKC inhibitor BIS for 1 h before stimulation with LPS prevented the phosphorylation of TRAM (Fig. 2*c*, lanes 7–12). Immunoblotting

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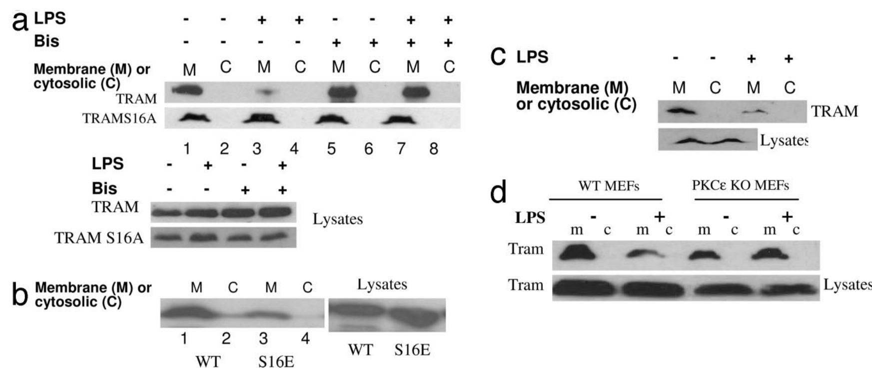

Fig. 3. Phosphorylation of TRAM causes it to dissociate from the membrane. Membrane fractionation, as described in *Methods*, was carried out on HEK293-TLR4 cells overexpressing FLAG-tagged TRAM or TRAMS16A that were preincubated with 10 μM BIS for 1 h before stimulation with 1 μg/ml LPS for 30 min (*a*), HEK293-TLR4 cells overexpressing FLAG-tagged TRAM or TRAMS16E (*b*), THP1 cells stimulated with 1 μg/ml LPS for 30 min (*c*), or PKCε$^{-/-}$ MEFs (PKCε KO MEFs) and PKCε$^{-/-}$ MEFs that had been reconstituted (WT MEFs) with PKCε overexpressing FLAG-tagged TRAM stimulated with 1 μg/ml LPS for 30 min (*d*). Results shown are representative of at least three experiments.

of lysates from PKCε$^{-/-}$ MEFs reconstituted with PKCε treated with LPS for 15 and 30 min revealed a band of the correct molecular weight as TRAM (Fig. 2*d Upper*, lanes 9 and 10). The phosphorylation occurred earlier in this cell type than in the THP1 cells; the effect was waning by 45 min (lane 11) and was not evident at 60 min (lane 12). Levels of total TRAM were not altered in the lysates over the time course (Fig. 2*d Lower*). Importantly, no band was detected in lysates generated from PKCε$^{-/-}$ MEFs (Fig. 2*d Upper*, lanes 1–6), and no bands were detected in TRAM-deficient cells, attesting to the specificity of the antibody (data not shown). Finally, treatment of PKCε-expressing MEFs with the TLR2 ligand macrophage-activating lipopeptide-2 (MALP2) or the TLR3 ligand poly(I·C) for 30 min had no effect (data not shown). Taken together, these studies confirm the LPS-dependent phosphorylation of TRAM on serine-16 by PKCε.

Levels of TRAM in the Membrane Are Reduced upon LPS Stimulation. The myristoylation of TRAM promotes membrane localization. It is known that certain myristoylated proteins dissociate from the membrane upon phosphorylation (17–19). We wished to investigate whether phosphorylation by PKCε would cause a redistribution of TRAM. FLAG-TRAM/pcDNA3.1 was transfected into HEK293-TLR4 cells, and these cells were then stimulated with or without LPS for 30 min. The cells were fractionated into membrane and cytosolic fractions. The amount of TRAM present in the membrane fraction was decreased upon LPS stimulation, suggesting that TRAM is disappearing from the membrane (Fig. 3*a*, first panel, compare lane 3 with lane 1). We could not detect TRAM in the cytosolic fraction. This effect was not because of degradation, because the levels of TRAM in the cell lysates remained constant (Fig. 3*a*, third panel). The depletion of TRAM from the membrane was PKCε-dependent, because the addition of the PKC inhibitor BIS 1 h before LPS stimulation caused FLAG-TRAM to remain in the membrane even after LPS stimulation (Fig. 3*a*, lane 7). When we tested for depletion of TRAMS16A, no depletion from the membrane was observed (Fig. 3*a*, second panel). We wished to see whether phosphorylation of serine-16 was sufficient to allow TRAM to leave the membrane. This serine was mutated to a glutamic acid, and this mutation caused a significant decrease in the amount of TRAM present in the membrane (Fig. 3*b*, compare lane 3 with lane 1), suggesting that the phosphorylation of TRAM on serine-16 causes depletion of TRAM from the membrane. We were also able to detect depletion of endogenous TRAM in THP1 cells treated with LPS (Fig. 3*c*). Finally, in PKCε$^{-/-}$ MEFs FLAG-TRAM did not become depleted from the membrane upon LPS stimulation (Fig. 3*d*). This evidence suggests that the phosphorylation of TRAM on serine-16 by PKCε is required for TRAM to be depleted from the membrane.

TRAM Phosphorylation by PKCε Is Required for TRAM Signaling. Having identified TRAM as a substrate for PKCε, the question then arose as to the functional importance of this response. We first tested the effect of the PKC inhibitor BIS on TRAM signal transduction. Overexpression of TRAM activated an NF-κB-linked reporter gene 10-fold over control levels (Fig. 4*a*). Pretreatment of cells with BIS inhibited this effect, with no effect being evident against MyD88. We next tested the ability of the TRAM mutants to activate IRF3 and also NF-κB-dependent reporter genes in HEK293 cells. As shown in Fig. 4*b*, mutations in serine-10 and serine-14 had no effect on the ability of TRAM to activate IRF3 or NF-κB (data not shown), suggesting that these serines are not vital for TRAM to function. Mutating serine-6 significantly reduced the ability of TRAM to activate IRF3 or NF-κB, whereas mutating serine-16 abolished the ability of TRAM to activate IRF3 or NF-κB. The lack of effect of the serine-6 mutant was likely to be due to the lack of myristoylation. TRAMS16A also inhibited the effect of WT TRAM on both IFN-stimulated response element (ISRE) (Fig. 4*b*, last histogram on the right) and NF-κB (data not shown).

TRAM-Dependent Pathways Are Disrupted in PKCε$^{-/-}$ MEFs. Previous studies in PKCε$^{-/-}$ cells revealed that induction of nitric oxide,

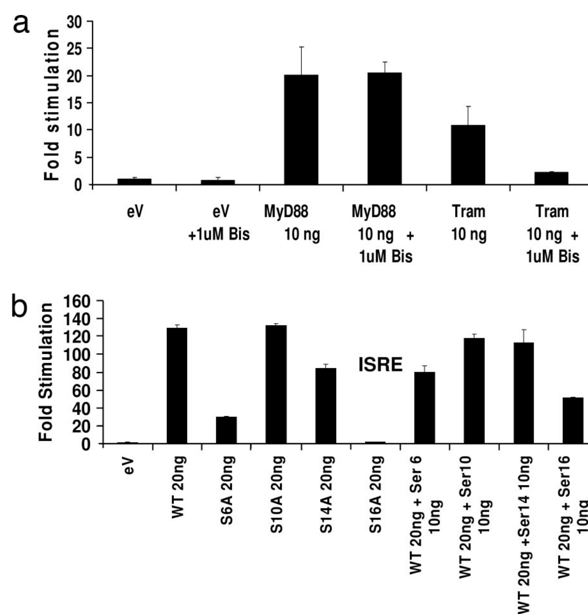

Fig. 4. The phosphorylation of TRAM by PKCε is vital for TRAM to function. (*a*) HEK293 cells, treated with or without 10 μM BIS for 2 h, were transiently transfected with FLAG-tagged TRAM, MyD88, or empty vector, together with the NF-κB luciferase reporter gene. (*b*) HEK293 cells were transiently transfected with WT TRAM and/or one of the four TRAM mutants, together with the ISRE–luciferase reporter gene. The data represents the mean fold stimulation of luciferase activity relative to control levels. Results shown are representative of at least three experiments.

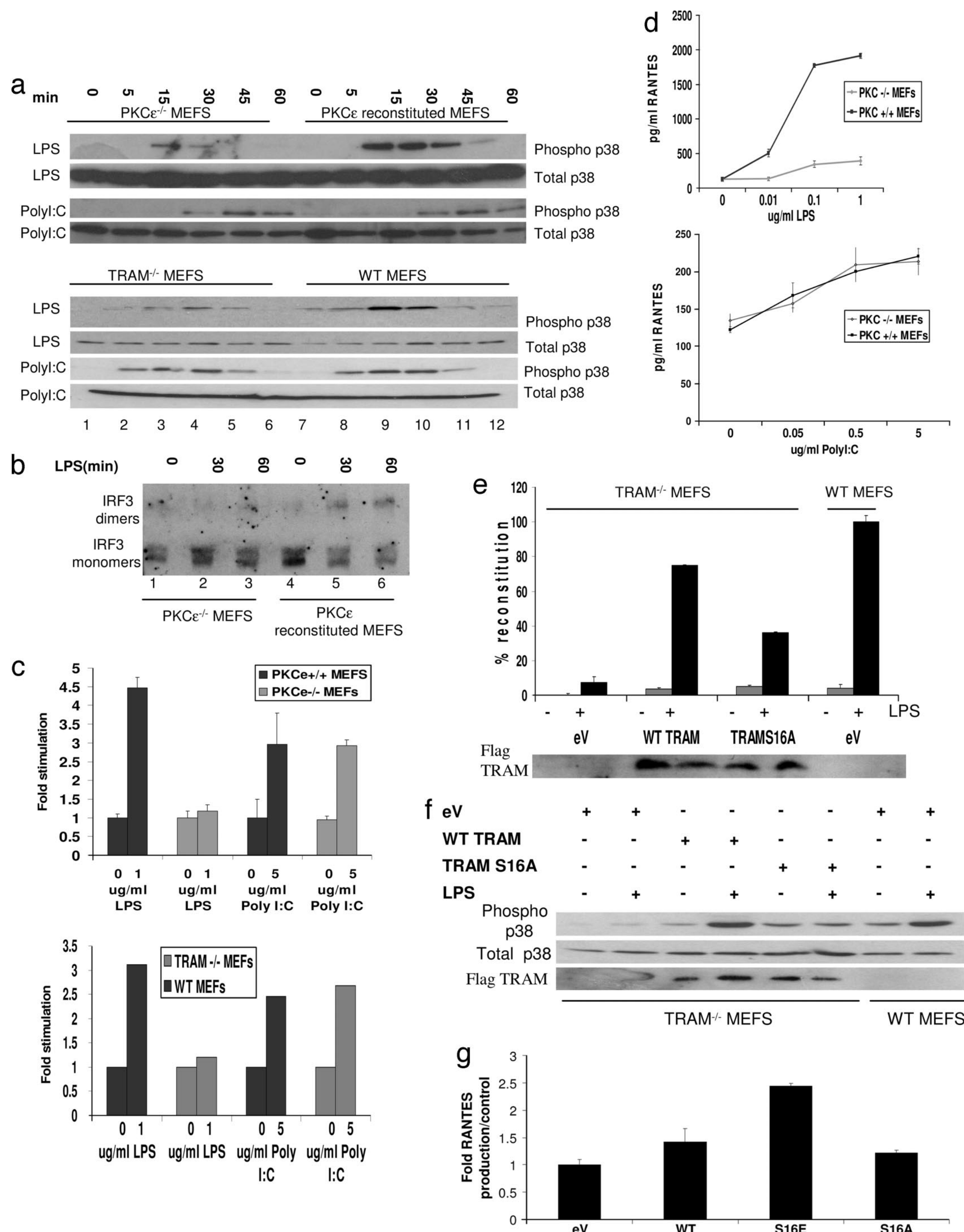

Fig. 5. TRAM and PKCε are both essential for complete LPS signaling. (*a*) Cells from PKC$\varepsilon^{-/-}$ MEFs and PKC$\varepsilon^{-/-}$ MEFs that had been reconstituted with PKCε (first four panels) or TRAM$^{-/-}$ MEFs and WT MEFs (last four panels) were stimulated for the indicated times with 1 μg/ml LPS or 5 μg/ml poly(I·C), and the lysates were immunoblotted for phosphorylated (tyrosine-180/182) and total p38. (*b*) PKC$\varepsilon^{-/-}$ MEFs and PKC$\varepsilon^{-/-}$ MEFs that had been reconstituted with PKCε were treated with 1 μg/ml LPS for the indicated times, run on a nonreducing PAGE gel, and immunoblotted for IRF3. (*c*) The ISRE–luciferase reporter gene and the control *Renilla* luciferase reporter gene were transfected into the above cell types. Twenty-four hours after transfection the cells were stimulated with 1 μg/ml LPS or 5 μg/ml poly(I·C) for 6 h. The data represent mean fold stimulation of luciferase activity relative to control levels. (*d*) PKC$\varepsilon^{-/-}$ MEFs and PKC$\varepsilon^{-/-}$ MEFs that had been reconstituted with PKCε were stimulated with the indicated concentrations of LPS or poly(I·C) for 24 h, and then a RANTES ELISA was performed. (*e* and *f*) WT TRAM, TRAMS16A, and empty vector were transfected into TRAM-deficient MEFs. (*e*) Twenty-four hours after transfection cells were stimulated with 1 μg/ml LPS for a further 24 h, and the culture supernatants were assayed for RANTES by ELISA. (*f*) Cells were stimulated with 1 μg/ml LPS for 30 min, and the lysates were assayed for p38 by Western blot. (*g*) WT TRAM, TRAMS16A, TRAMS16E, and empty vector were transfected into TRAM-deficient MEFs. Twenty-four hours after transfection the culture supernatants were assayed for RANTES by ELISA. Results shown are representative of at least three experiments.

TNF, and IL-12, as well as lethality induced by LPS, are impaired in PKC$\varepsilon^{-/-}$ mice (7). These responses are TRAM-dependent (12–14). We therefore tested TRAM-dependent signaling in PKC$\varepsilon^{-/-}$ MEFs. In both TRAM$^{-/-}$ MEFs and PKC$\varepsilon^{-/-}$ MEFs the phosphorylation of p38 in cells treated with LPS was significantly reduced in comparison to their corresponding WT

MEFs (Fig. 5*a*, first panel, compare lanes 9–11 with lanes 3–5). Importantly, there was no reduction in p38 phosphorylation in response to poly(I·C) in PKC$\varepsilon^{-/-}$ MEFs or TRAM$^{-/-}$ MEFs (Fig. 5*a*, third panel, compare right and left sides). We also tested the activation of IRF3 by LPS as indicated by its dimerization. IRF3 dimerization induced by LPS was reduced in PKC$\varepsilon^{-/-}$ MEF relative to MEFs expressing PKCε (Fig. 5*b*, compare lanes 2 and 3 with lanes 5 and 6). As shown by Yamamoto *et al.* (14), this response was also impaired in TRAM-deficient MEFs in response to LPS. We tested activation of an IRF3-linked reporter gene, and this was impaired in LPS-treated PKC$\varepsilon^{-/-}$ MEFs but was normal in poly(I·C)-treated PKC$\varepsilon^{-/-}$ MEFs (Fig. 5*c Upper*). Similar results were obtained in TRAM-deficient MEFs (Fig. 5*c Lower*). We next tested induction of RANTES as a readout for the TRAM pathway. In TRAM$^{-/-}$ MEFs the levels of RANTES produced in response to LPS stimulation was dramatically reduced in comparison to the corresponding WT MEFs (data not shown). The level of RANTES production in response to poly(I·C) was not affected (data not shown). Importantly, this response was also impaired in PKC$\varepsilon^{-/-}$ cells. As shown in Fig. 5*d Upper*, induction of RANTES by LPS was impaired relative to PKCε-expressing cells. There was no difference in the response to poly(I·C) when both cells types were compared (Fig. 5*d Lower*).

TRAMS16A Is Unable to Fully Reconstitute Signaling in TRAM-Deficient Cells. The clear impairment in TRAM-dependent responses after LPS treatment in PKC$\varepsilon^{-/-}$ cells, with the same responses being intact in poly(I·C)-treated cells, coupled with impaired signaling by TRAMS16A, strongly suggested that TRAM phosphorylation by PKCε is essential for TRAM function. To provide further evidence for this effect we examined the ability of TRAMS16A and TRAMS16E to reconstitute signaling in TRAM-deficient MEFs. Treatment of WT MEFs with LPS induced RANTES production, whereas treatment of TRAM$^{-/-}$ MEFs with LPS caused little or no induction of RANTES production (Fig. 5*e*). The response of the TRAM-deficient cells could be reconstituted with WT TRAM. Significantly, TRAMS16A was less capable of reconstituting the signal. Similarly, the phosphorylation of p38 in TRAM-deficient cells upon LPS stimulation was reconstituted with overexpression of WT TRAM. TRAMS16A could not reconstitute this signal (Fig. 5*f*). The TRAMS16E mutant, which mimics phosphorylated TRAM, was active even in the absence of LPS, inducing RANTES production when overexpressed (Fig. 5*g*). This finding further supported our conclusion that phosphorylation of TRAM on serine-16 by LPS is vital for it to function. We therefore conclude that phosphorylation of serine-16 by PKCε must be required for TRAM to function normally upon LPS stimulation.

Discussion

Before the discovery of several of the key signaling proteins required for LPS-mediated responses in macrophages, a role for PKCε was indicated from studies involving PKC$\varepsilon^{-/-}$ mice and PKC inhibitors (7, 21). Macrophages from PKC$\varepsilon^{-/-}$ mice treated with LPS showed reduced expression of several cytokines involved in the innate immune response (7). However, an important unanswered question was the point on the TLR4 signaling pathway targeted by PKCε. In this article we have discovered a novel target for PKCε. TRAM, a TIR domain-containing adapter used solely by TLR4 to initiate a signal after LPS stimulation, is phosphorylated by PKCε during LPS signaling. This process is required for TRAM to signal. We have thus identified a key process activated by TLR4 and pinpointed an important function for PKCε.

TRAM is myristoylated, and the function of this modification is probably to localize TRAM to the membrane such that it can be phosphorylated by PKCε, which acts in the membrane. TRAM clearly undergoes phosphorylation by PKCε during TLR4 signaling. We identified serine-16 as a key phosphoaccepting amino acid in TRAM, and we used a phosphospecific antibody to detect the increase in phosphorylation upon treatment of cells with LPS. This response serves as a useful readout for TLR4 signaling, because TLR2 and TLR3, neither of which signals via TRAM, were without effect. The phosphorylation on serine-16 upon LPS stimulation is transient. Serine-6 also appears to be phosphorylated, but its positioning in the myristoylation consensus sequence made it difficult to analyze, as we have found that TRAM lacking a myristate group is inactive (27). One consequence of phosphorylation of myristoylated proteins near the site of myristoylation is translocation from the membrane (24). We have shown that TRAM is depleted from the membrane upon phosphorylation by PKCε. This process is PKCε-dependent, because it is blocked by the pan PKC inhibitor BIS and does not occur in PKC$\varepsilon^{-/-}$ cells. The exact consequence of this event and the exact location of TRAM upon depletion from the membrane have yet to be elucidated. Whether this process is required for TRAM to function or is a down-regulatory mechanism is still unclear; however, the response serves as another indicator of the targeting of TRAM by PKCε and is likely to be functionally important, possibly allowing TRAM to interact with and activate downstream signals, such as activation of TANK-binding kinase 1 (TBK-1), which would lead to IRF3 activation (13). We are currently testing this possibility. Equally, however, TRAM may be phosphorylated by PKCε, becomes activated, and is then depleted from the membrane. Despite much effort, we have been unable to determine the precise function of TRAM phosphorylation by PKCε, only that it is required for TRAM to function.

Previous studies in PKC$\varepsilon^{-/-}$ cells revealed an impairment in the induction of several cytokines, such as IFNβ, which, similar to RANTES, is TRAM-dependent. It is therefore likely that the molecular basis for the defect in PKC$\varepsilon^{-/-}$ mice is impaired phosphorylation of TRAM by PKCε. The TRAM/Trif-dependent LPS signals, such as IRF3 activation and RANTES production, are induced by Trif alone during TLR3 activation. Importantly, these TLR3-dependent responses are not impaired in PKC$\varepsilon^{-/-}$ cells, suggesting that PKCε acts upstream of Trif only in TLR4 signaling. Clear evidence for TRAM being the key target of PKCε comes from reconstitution experiments in TRAM-deficient cells where the TRAMS16A mutant was less able to reconstitute LPS responses in comparison to WT TRAM, whereas the TRAMS16E mutant was active even in the absence of LPS. The residual activity of the TRAMS16A mutant may be due to the fact that serine-6 is still being phosphorylated, which might be sufficient for TRAM to signal by means of translocation from the membrane or conformational change. This effect would be more efficient when both serine-6 and serine-16 are phosphorylated. Finally, in terms of upstream regulation, a role for phospholipase C and DAG in LPS signaling has been indicated for some time (25, 26). Our study provides a possible target for these molecules in LPS signaling. DAG may activate PKCε, leading to the phosphorylation and activation of TRAM. Further research is needed to determine whether this is the case.

In conclusion, our study provides a molecular explanation for the role of PKCε in LPS signaling, identifying TRAM as a key target in this pathway. We have therefore elucidated an important process in TLR4 signaling that might lend itself to therapeutic manipulation in conditions such as septic shock.

Methods

Cloning TRAM. TRAM cDNA was generated by performing RT-PCR on a spleen mRNA library (BD Biosciences). Reverse transcription was carried out by using the Moloney murine leukemia virus reverse transcriptase enzyme (Promega) following the manufacturer's instructions. Five microliters of this reaction was used as a template for a PCR using specific primers to the 5′ and 3′ ends of TRAM. TRAM was then cloned into the GST expression vector pGEX-KG, a FLAG tag was added to the 3′ end of TRAM, and

this gene was cloned into the mammalian expression vector pCDNA3.1.

Site-Directed Mutagenesis of TRAM. The QuikChange site-directed mutagenesis kit (Stratagene) was used to mutate certain bases in the TRAM gene. The manufacturer's instructions were followed using primers containing the desired mutation.

Luciferase Reporter Gene Assays. HEK293 or HEK293-TLR4 cells were seeded in 96-well plates at a density of 1×10^5 cells per milliliter. The next day the cells were transfected with 80 ng of the NF-κB or IRSE–luciferase reporter gene along with 40 ng of the *Renilla* luciferase internal control plasmid. After 24 h the cells were lysed in passive lysis buffer (Promega), and reporter gene activity was measured by using a luminometer. The data were expressed as mean fold stimulation relative to control levels.

Kinase Assay. GST-TRAM was overexpressed in BL21(DE3) cells and purified by using glutathione Sepharose beads. THP1 cells were seeded at 2×10^5 cells per milliliter, and the next day 30 ml of cells was treated with LPS for 30 min and then lysed in 1 ml of buffer [10% glycerol (vol/vol)/50 mM NaF/20 mM Tris·Cl, pH 8.0/2 mM EDTA/137 mM NaCl/1% Nonidet P-40/1 mM PMSF/10 μg/ml leupeptin/1 mM Na_3VO_4]. The lysate was added to 50 μl of the purified GST-TRAM and incubated for 2 h at 4°C. The beads were spun down at $800 \times g$ for 5 min and washed three times in kinase buffer [20 mM Hepes, pH 7.5/10 mM $Mg(OAc)_2$/0.03% Triton X-100/100 μg/ml phosphatidylserine/20 mM β-glycerol phosphate/0.01% (wt/vol) leupeptin/100 μM Na_3VO_4]. These beads were resuspended in 30 μl of kinase buffer containing 20 μM unlabeled ATP and 5 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP and incubated at 37°C for 30 min. A total of 20 μl of sample buffer [50 mM Tris·Cl, pH 6.8/10% glycerol (vol/vol)/2% SDS (wt/vol)/0.1% bromophenol blue (wt/vol)/5% 2-mercaptoethanol] was added, and the sample was boiled for 5 min. The sample was then run on a 10% SDS/PAGE gel and transferred to nitrocellulose. The nitrocellulose was exposed on x-ray film.

Membrane Fractionation. HEK293-TLR4 cells were seeded at 1×10^5 cells per milliliter overnight and then transfected with the appropriate plasmids. Twenty-four hours after transfection the cells were treated as directed in *Results* and then scraped into 300 μl of membrane buffer (20 mM Tris, pH 7.5/10 mM $MgCl_2$/1 mM EDTA/250 μM sucrose/200 μM PMSF). The cells were lysed by using 30 strokes of a Dounce homogenizer and spun in hardwall Beckman tubes at $425,000 \times g$ for 1 h at 4°C. The supernatant, i.e., the cytosolic fraction, was removed to a fresh tube, and the pellet, i.e., the membrane fraction, was resuspended in 50 μl of sample buffer [50 mM Tris·Cl, pH 6.8/10% glycerol (vol/vol)/2% SDS (wt/vol)/0.1% bromophenol blue (wt/vol)/5% 2-mercaptoethanol]. The cytosolic fraction was concentrated down to 50 μl by using a Centricon YM-10 (Millipore). The samples were run on a 12% SDS/PAGE gel.

RANTES ELISA. The indicated cells were seeded at 1×10^5 cells per milliliter overnight in 24-well plates and then transfected with the appropriate plasmids. Twenty-four hours after transfection the cells were treated with the appropriate stimuli for 24 h. By using a 1:5 dilution of the supernatant as the sample, a RANTES ELISA was performed using the mouse RANTES kit from R & D Systems following the manufacturer's instructions.

IRF3 Dimerization Assay. The appropriate cells were seeded at 2×10^5 cells per milliliter overnight and then treated with the appropriate stimuli. The cells were washed in PBS and scraped into 100 μl of nonreducing sample buffer [50 mM Tris·Cl, pH 6.8/10% glycerol (vol/vol)/0.1% bromophenol blue (wt/vol)/5% 2-mercaptoethanol]. A total of 20 μl of each sample was run on a nonreducing PAGE gel, transferred onto nitrocellulose, and immunoblotted for IRF3.

Detection of TRAM Phosphorylation Using the Phosphoserine Antibody. FLAG-TRAM was transfected into HEK293-TLR4 cells by using GeneJuice and left for 24 h. The cells were then lysed in 600 μl of lysis buffer [10% glycerol (vol/vol)/50 mM NaF/20 mM Tris·Cl, pH 8.0/2 mM EDTA/137 mM NaCl/1% Nonidet P-40/1 mM PMSF/10 μg/ml leupeptin/1 mM Na_3VO_4]. A total of 20 μl of FLAG agarose beads (Sigma) was added to the lysates and incubated for 1 h at 4°C. The beads were spun at $800 \times g$ for 5 min and then washed three times in lysis buffer and resuspended in 50 μl of sample buffer [50 mM Tris·Cl, pH 6.8/10% glycerol (vol/vol)/2% SDS (wt/vol)/0.1% bromophenol blue (wt/vol)/5% 2-mercaptoethanol]. The samples were run on 12% SDS/PAGE gels, transferred onto nitrocellulose, and immunoblotted for TRAM and phosphoserine.

Production of a Phosphospecific TRAM Antibody. FabGennix (Frisco, TX), immunized a rabbit with a synthetic peptide corresponding to amino acids 7–21 of TRAM (KINSCPLSLSWGKRH) with a phosphoserine incorporated instead of the serine at amino acid 16. The phosphospecific antibodies were then affinity purified from the blood by using the phosphopeptide. The validity of the antibody was confirmed when the band predicted to the phosphorylated TRAM was not present in samples taken from TRAM-deficient MEFs (data not shown).

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