

Comment on “PDK1 Nucleates T Cell Receptor–Induced Signaling Complex for NF- κ B Activation”

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We observe that protein kinase C θ (PKC θ) is phosphorylated on the activation loop at threonine 538 (Thr-538) before T cell activation. Our results are inconsistent with the conclusions of Lee *et al.* (Reports, 1 April 2005, p. 114) that the Thr-538 phosphorylation of PKC θ is regulated by T cell receptor activation. Other mechanisms, such as autophosphorylation of Thr-219, might orchestrate the cellular function of PKC θ in T cells.

The serine/threonine kinase PKC θ plays a crucial role in T cell activation and has been firmly established as a central mediator of T cell signaling in *in vitro* as well as in *in vivo* models (1–3). The catalytic activity of PKC θ is controlled, in part, by phosphorylation of the activation-loop residue (Thr-538) on the kinase domain by the upstream enzyme 3-phosphoinositide-dependent kinase 1 (PDK1) or a related enzyme (2). However, the phosphorylation status of PKC θ at Thr-538 in T lymphocytes has not been fully characterized.

In the work of Lee *et al.* (4), mostly based on *in vitro* studies with the Jurkat human T cell line, PDK1 appeared to inducibly phosphorylate PKC θ at Thr-538 after T cell stimulation with antibodies to CD3 and CD28. This inducible phosphorylation was a prerequisite for activation of I κ B kinase and nuclear factor κ B (IKK/NF- κ B). The authors thus proposed that induced phosphorylation of PKC θ at Thr-538 plays a key role in its isotype-specific activation and cellular function following T cell activation.

Results independently obtained in our four laboratories are not consistent with the conclusions described by Lee *et al.* that phosphorylation of PKC θ at Thr-538 is largely regulated by T cell receptor (TCR)–

mediated stimulation. In contrast, we reproducibly observe constitutive phosphorylation of PKC θ at Thr-538, using phospho(p)–Thr538-specific antibodies from independent sources, in both primary mouse and human T cells and Jurkat T cell lines, as illustrated below.

We observed that the (p)Thr-538 PKC θ antiserum from Cell Signaling Technology (CST) reacted strongly with endogenous PKC θ in resting mouse CD3⁺ cells and that no additional phosphorylation on Thr-538 could be induced

not further induced by stimulation (Fig. 2A). The strong immunoreactivity against the recombinant PKC θ wild-type protein in transfected Jurkat cells proved to be site-specific, because no immunoreactivity above the endogenous situation was seen when a PKC θ T538A mutant was expressed, thus further validating the (p)T538 antiserum from CST. Constitutive phosphorylation on T538 is not an artifact of transfection or overexpression, because endogenous PKC θ in Jurkat cells displayed an identical pattern of phosphorylation on the activation loop when compared with transfected cells (Fig. 3).

The experiments described above were performed in the presence of 10% serum-containing medium. To ensure that the high basal level of phosphorylated PKC θ was not due to high serum levels (and therefore masking phosphorylation induced by antibodies to CD3 and CD28), Jurkat cells were cultured for 72 hours in low-serum conditions (0.5%) before stimulation with antibodies to CD3 and CD28. Culturing the cells in low levels of serum did not alter PKC θ phosphorylation (Fig. 2B); Thr-538 was still robustly phosphorylated in low-serum conditions, and stimulation with antibodies to CD3 and CD28 did not increase the levels of phosphorylation. Strong and inducible MAPK phosphorylation was achieved upon CD3/CD28 ligation under all serum conditions, therefore validating our activation protocol.

Our results demonstrate that PKC θ is phosphorylated at Thr-538 in the activation loop before T cell activation and that Thr-538 phosphorylation is not an immediate activation step but rather a prerequisite for the catalytic competence of the enzyme. Phosphorylation of the activation loop primes the enzyme for activation in response to second messengers such as diacylglycerol or phorbol esters. This is consistent with the finding that a T538A PKC θ mutant cannot be activated by phorbol esters

in vitro (6). Instead, phosphorylation of Thr-538 on PKC θ is required for correctly aligning the residues that are involved in catalysis and, as such, ensures competency to respond to second messengers *in vivo* (7). In support of this interpretation, conventional PKC (cPKC) isotypes are first phosphorylated in the activation loop by the upstream kinase PDK1 (or a related enzyme) in a cotranslational manner. Phosphorylation of the activation loop permits phosphorylation on the turn and hydrophobic motif sites. Phosphorylation does not activate the enzyme but allows cPKC isotypes to adopt a mature and stable conformation, ready to

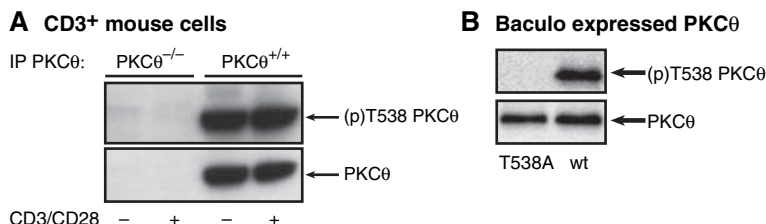


Fig. 1. (A) Constitutive activation-loop phosphorylation of PKC θ in CD3⁺ T cells. Murine CD3⁺ T cells were negatively selected and purified from lymph nodes and spleen of wild-type and PKC $\theta^{-/-}$ mice. PKC θ was immunoprecipitated (IP) from resting (–) or 1-hour stimulated (+; by solid-phase antibodies to CD3 and CD28) mouse CD3⁺ cells, employing the PKC θ -specific pAb from Santa Cruz. The Thr-538 phosphostatus was determined in an immunoblot, employing the PKC θ (p)Thr-538 specific pAb from CST. **(B)** Phosphopeptide antibody to Thr-538 reacts in an epitope-specific manner. Wild-type and T538A mutant PKC θ proteins were expressed in baculovirus and purified to homogeneity and tested *in vitro* with the (p)Thr-538 PKC θ antiserum from CST. Immunoreactivity was phosphosite-specific for Thr-538 of PKC θ as no immunoreactivity was observed with the PKC θ T538A mutant protein.

upon stimulation with antibodies to CD3 and CD28 (Fig. 1A). As specificity controls for the (p)Thr-538 antiserum, we demonstrate that PKC θ -deficient CD3⁺ T cell lysates derived from our established PKC $\theta^{-/-}$ mouse line (5) did not reveal any band (Fig. 1A, left two lanes). In addition, the (p)Thr-538 antiserum did not recognize a purified PKC θ mutant in which the Thr-538 residue was exchanged for alanine (T538A) (Fig. 1B).

Studies in the Jurkat human T cell line confirmed that phosphorylation on Thr-538 is constitutive; again we detected a high basal Thr-538 phosphorylation on PKC θ , which was

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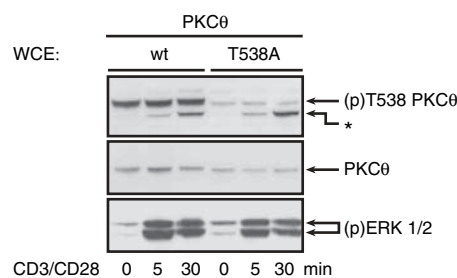
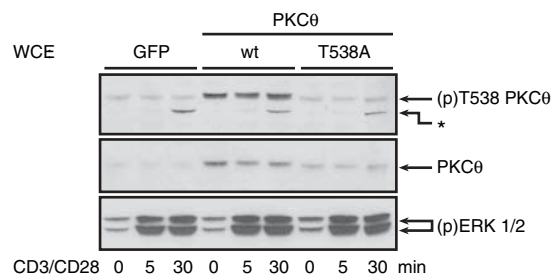
A Jurkat (10% FCS)

Fig. 2. Constitutive activation-loop phosphorylation of PKC θ in Jurkat T cells. Jurkat cells were maintained in 10% FCS (**A**) or 0.5% FCS for 48 hours (**B**) and then transiently transfected with wild-type PKC θ and T538A mutant. Serum-starved cells were also transfected with a GFP inert protein control and maintained in 0.5% FCS at all times. After 24 hours, cells were activated with solid-phase antibodies to CD3 and CD28 for various times, as indicated. The Thr-538 phospho-status was determined by immunoblotting of whole cell extracts (WCE). As a loading control, the membranes were stripped and reprobed with antibodies to total PKC θ . A nonspecific, albeit inducible 72-kD protein band recognized by the (p)Thr-538 antiserum from CST is marked with an asterisk. As an internal activation marker, the lysates were immunostained in parallel with an antibody to phospho-MAPK that detects dually phosphorylated (active) extracellular signal-regulated kinase 1 (ERK1) and ERK2.

B Jurkat (0.5% FCS)

are not known at present. The discrepancies may be attributable to different origins of the Jurkat cells used in the respective laboratories. Still, the phospho-status of endogenous PKC θ in the signaling pathways of primary CD3⁺ T cells strongly argues that stimulation-dependent induction does not occur at Thr-538 on PKC θ .

References and Notes

1. S. L. Tan, P. J. Parker, *Biochem. J.* **376**, 545 (2003).
2. G. Baier, *Immunol. Rev.* **192**, 64 (2003).
3. B. J. Marsland, T. J. Soos, G. Spath, D. R. Littman, M. Kopf, *J. Exp. Med.* **200**, 181 (2004).
4. K. Y. Lee, F. D'Acquisto, M. S. Hayden, J. H. Shim, S. Ghosh, *Science* **308**, 114 (2005).
5. C. Pfeifferhofer *et al.*, *J. Exp. Med.* **197**, 1525 (2003).
6. Y. Liu, C. Graham, A. Li, R. J. Fisher, S. Shaw, *Biochem. J.* **361**, 255 (2002).
7. Z. B. Xu *et al.*, *J. Biol. Chem.* **279**, 50401 (2004).
8. A. C. Newton, *Biochem. J.* **370**, 361 (2003).
9. N. Thuille *et al.*, *EMBO J.* **24**, 3869 (2005).
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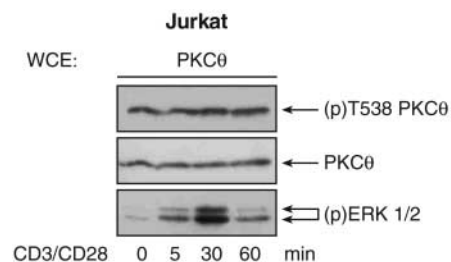


Fig. 3. Constitutive activation-loop phosphorylation of endogenous PKC θ in Jurkat T cells. Cells were maintained in 10% FCS at all times and stimulated with solid-phase antibodies to CD3 and CD28 for various times, as indicated. Thr-538 phospho-status determination, loading control, and internal activation marker were the same as described in Fig. 2.

be activated by diacylglycerol or phorbol esters (8).

In the light of our data, a new interpretation regarding the induction of (p)Thr-538 on PKC θ observed by Lee *et al.* (4) is necessary. We conclude that other mechanisms, independent of TCR-mediated (p)Thr-538 induction of PKC θ , are playing the observed role in the correct targeting and cellular function of PKC θ upon antigen receptor ligation. Along this line, at least one additional activation-induced autophosphorylation site at Thr-219 appears to exist between the tandem C1 domains of the regulatory fragment in PKC θ . Thr-219 phosphorylation plays an important role in the correct cellular function of PKC θ upon antigen receptor ligation (9).

The reasons for the differences between our results and the previously published report (4)