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

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We observe that protein kinase C θ (PKCθ) 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 PKCθ is regulated by T cell receptor activation. Other mechanisms, such as autophosphorylation of Thr-219, might orchestrate the cellular function of PKCθ in T cells.

The serine/threonine kinase PKCθ plays a crucial role in T cell activation and has been firmly established as a central mediator of T cell signaling in vitro as well as in vivo models (1–3). The catalytic activity of PKCθ 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 PKCθ 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 PKCθ at Thr-538 after T cell stimulation with antibodies to CD3 and CD28. This inducible phosphorylation was a prerequisite for activation of IkB kinase and nuclear factor κB (IKK/NF-κB). The authors thus proposed that induced phosphorylation of PKCθ 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 PKCθ at Thr-538 is largely regulated by T cell receptor (TCR)–mediated stimulation. In contrast, we reproducibly observe constitutive phosphorylation of PKCθ 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 PKCθ antiserum from Cell Signaling Technology (CST) reacted strongly with endogenous PKCθ in resting mouse CD3+ cells and that no additional phosphorylation on Thr-538 could be induced upon stimulation with antibodies to CD3 and CD28 (Fig. 1A). As specificity controls for the (p)Thr-538 antiserum, we demonstrate that PKCθ-deficient CD3+ T cell lysates derived from our established PKCθ−/− 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 PKCθ 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 PKCθ, which was not further induced by stimulation (Fig. 2A). The strong immunoreactivity against the recombinant PKCθ wild-type protein in transfected Jurkat cells proved to be site-specific, because no immunoreactivity above the endogenous situation was seen when a PKCθ T538A mutant was expressed, thus further validating the (p)T538 antiseraum from CST. Constitutive phosphorylation on T538 is not an artifact of transfection or overexpression, because endogenous PKCθ 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 PKCθ 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 PKCθ 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 PKCθ 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 PKCθ mutant cannot be activated by phorbol esters in vitro (6). Instead, phosphorylation of Thr-538 on PKCθ 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...
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 PKCθ observed by Lee et al. (4) is necessary. We conclude that other mechanisms, independent of TCR-mediated (p)Thr-538 induction of PKCθ, are playing the observed role in the correct targeting and cellular function of PKCθ 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 PKCθ. Thr-219 phosphorylation plays an important role in the correct cellular function of PKCθ upon antigen receptor ligation (9).

The reasons for the differences between our results and the previously published report (4) are not known at present. The discrepancies may be attributable to different origins of the Jurkat cells used in the respective laboratories. Still, the phosho-status of endogenous PKCθ in the signaling pathways of primary CD3+ T cells strongly argues that stimulation-dependent induction does not occur at Thr-538 on PKCθ.

Fig. 2. Constitutive activation-loop phosphorylation of PKCθ 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 PKCθ 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 striped and reprobed with antibodies to total PKCθ. 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.

Fig. 3. Constitutive activation-loop phosphorylation of endogenous PKCθ 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.

References and Notes
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