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Phosphotyrosine-dependent interaction between the kinases PKC θ and Zap70 promotes proximal TCR signaling

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Abstract

Protein kinase C- θ (PKC θ) is an important component of proximal T cell receptor (TCR) signaling. We previously identified the amino-terminal C2 domain of PKC θ as a phosphotyrosine (pTyr)-binding domain. Using a mutant form of PKC θ that cannot bind pTyr (PKC θ^{HR2A}), we show that pTyr binding by PKC θ was required for TCR-induced T cell activation, proliferation, and Th2 differentiation, but not for T cell development. Using tandem mass spectrometry and co-immunoprecipitation, we identified the kinase ζ -associated protein kinase of 70 kDa (Zap70) as a ligand of the PKC θ pTyr-binding pocket. Tyr¹²⁶ of Zap70 directly bound to PKC θ , and the interdomain B residues Tyr³¹⁵ and Tyr³¹⁹ were indirectly required for binding to PKC θ , reflecting their role in promoting the open conformation of Zap70. PKC θ^{HR2A} -expressing CD4⁺ T cells displayed strong defects not only in known PKC θ -dependent signaling events such as nuclear factor κ B (NF- κ B) activation and Th2 differentiation, but also had a defect in full activation of Zap70 itself and in the activating phosphorylation of linker of activation of T cells (LAT) and phospholipase C- γ 1 (PLC γ 1), signaling proteins that are traditionally considered to be activated PKC-independently. These findings demonstrate that PKC θ plays an important role in a positive

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SUPPLEMENTARY MATERIALS

Fig. S1. Normal T cell development in PKC θ^{HR2A} BM chimeric mice

Fig. S2. Impaired activation of peripheral T cells expressing PKC θ^{HR2A}

Fig. S3. Defective early TCR signaling in primary CD4⁺ T cells from *Prkcd*^{-/-} B6 mice

Fig. S4. Effect of HR2A-mutated PKC θ on Zap70 recruitment to TCR ζ and on formation of the PKC θ -CARMA1 complex

Table S1. Antibodies and other reagents used in this study

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: Mass spectrometry data have been deposited in the UCSD MassIVE database, <https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp>, with accession number MSV000083562. All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. Materials used in this study are available upon request.

feedback regulatory loop that modulates TCR-proximal signaling and, moreover, provide a mechanistic explanation for earlier reports that documented an important role for PKC θ in T cell Ca²⁺ signaling. This PKC θ -Zap70 interaction could potentially serve as a promising and highly selective immunosuppression drug target in autoimmunity and organ transplantation.

INTRODUCTION

T lymphocytes are a key component of the adaptive immune system. Protein kinase C- θ (PKC θ), which we have studied extensively (1), is found predominantly in T lymphocytes, where it plays non-redundant roles in cell activation, differentiation, proliferation, and survival (2, 3). PKC θ is unique among the PKC enzymes present in T cells because it undergoes peptide-MHC (pMHC)-induced translocation to the central region of the supramolecular activation cluster (cSMAC) region of the mature T cell immunological synapse (IS) (4). There it colocalizes with the T cell costimulatory molecule CD28 in a ring surrounding the central TCR (5). PKC θ is not globally required for all T cell-mediated responses but, rather, has a selective role, being required for Th2 (6, 7) and Th17 (8, 9) responses, allograft rejection (10), and graft-versus-host disease (GVHD) (11, 12), but not (or substantially less) for antiviral responses mediated by Th1 cells and cytotoxic T lymphocytes (12–15) or for graft-versus-leukemia responses (11, 12). In contrast to the positive regulatory role of PKC θ in T effector (Teff) cells, PKC θ negatively regulates the suppressive function of regulatory T (Treg) cells (16, 17). The molecular basis for the selective and context-dependent functions of PKC θ in T cells is unclear. Given the important and selective functions of PKC θ in T cells, substantial effort has been dedicated to developing selective PKC θ inhibitors for the treatment of autoimmune diseases, GVHD, and transplant patients (11, 18–20). However, it has generally been difficult to develop clinically useful, specific PKC θ catalytic inhibitors (and inhibitors of other PKC family members in general), likely due to the similarity between PKC family members, the multiple regulatory functions that PKC enzymes play in many cells and tissues, and the potential functional redundancy among PKC family members (21–23). Thus, a better understanding of the mechanisms that regulate PKC θ activation and downstream effector functions is needed.

The intracellular localization, activation, and effector functions of PKC θ are regulated by complex mechanisms, including phosphorylation (24–29), sumoylation (30), protein-protein interactions (2, 3, 31), intrinsic regulation by its regulatory domains (32–34), and catalytic activity (33). We have demonstrated the mechanism and importance of the localization of PKC θ to the cSMAC region of the IS by showing that the V3 (hinge) region between the regulatory and catalytic domains of PKC θ binds to the cytoplasmic tail of CD28, likely through the kinase Lck, and this association is critical for the downstream functions of PKC θ in T cells (31). We also demonstrated that, similar to its nearest relative within the PKC family, PKC δ (35), the N-terminal C2-like domain of PKC θ is a phosphotyrosine (pTyr)-binding domain (36). Nevertheless, the biological relevance of this function of PKC θ , as well as the identity of the pTyr-containing protein(s) that associate(s) with the PKC θ C2 domain in TCR-stimulated T cells remains to be determined. To address these unresolved questions, we functionally characterized PKC θ -deficient T cells reconstituted with a PKC θ

mutant that is incapable of pTyr binding and used mass spectrometry (MS) to identify a pTyr-containing ligand of the PKC θ C2 domain.

Here, we demonstrate that pTyr binding to the PKC θ C2 domain is required for full TCR-induced, PKC θ -dependent downstream signaling and effector functions, identify the ζ chain-associated protein kinase of 70 kDa (Zap70) as a PKC θ -interacting protein, and demonstrate a bidirectional regulatory interaction between PKC θ and Zap70 that, when disrupted, causes defects in early TCR signaling events that were traditionally considered to be independent of PKC θ . This PKC θ -Zap70 interaction could provide opportunities for the development of clinically useful allosteric inhibitors of PKC θ functions and, therefore, selective immunosuppression with less adverse side effects.

RESULTS

The pTyr-binding function of the C2 domain is required for PKC θ downstream signaling

PKC θ belongs to the “novel” subfamily of PKCs (nPKCs), which do not require Ca²⁺ for activation, and its nearest relative within this subfamily is PKC δ , with the C2 domains of these two PKCs displaying a substantial homology not shared by other PKCs (Fig. 1A). A previous study demonstrated that the PKC δ C2 domain was a pTyr-binding domain (35) that is structurally distinct from other known pTyr-binding domains, namely, the SH2 and PTB domains (37). Sequence alignment of the C2 domains of human and mouse PKC θ , PKC δ , PKC ϵ , and PKC η showed that critical residues in the pTyr-binding pocket of the PKC δ C2 domain (Lys⁴⁸, His⁶² and Arg⁶⁷) are conserved with PKC θ , whereas only one of these, Arg⁷⁹, is found PKC ϵ but not in PKC η (Fig. 1A). We previously demonstrated that the PKC θ C2 domain bound a pTyr-containing synthetic peptide and, furthermore, that mutation of His⁶³ or Arg⁶⁸ impaired the ability of PKC θ to stimulate a nuclear factor κ B (NF- κ B) reporter gene in transfected Jurkat T cells (36). To more rigorously explore the importance of pTyr binding for PKC θ 's TCR-induced downstream functions, we mutated both His⁶³ and Arg⁶⁸ in the C2 domain to alanine (HR2A mutant) and conducted a detailed functional analysis of PKC θ -deficient (*Prkcd*^{-/-}) CD4⁺ T cells reconstituted with this mutant.

Primary CD4⁺ T cells from *Prkcd*^{-/-} mice transduced with retrovirus (RV) expressing green fluorescent protein (GFP)-tagged wild-type PKC θ (PKC θ ^{WT}) or HR2A-mutated PKC θ (PKC θ ^{HR2A}) produced similar amounts of PKC θ protein (Fig. 1, B and C). Following a rest period, cells transduced with either PKC θ ^{WT} or PKC θ ^{HR2A} displayed very low abundance of the surface molecules CD25 and CD69, both of which are increased upon T cell activation, in the absence of restimulation. However, costimulation with monoclonal antibodies (mAbs) recognizing the TCR-associated CD3 complex and CD28 strongly increased the abundance of these two activation markers in the cells expressing PKC θ ^{WT}; in contrast, CD25 and CD69 abundance increased less in CD4⁺ T cells expressing PKC θ ^{HR2A} (Fig. 1D). The functional defects in PKC θ ^{HR2A}-expressing cells were more pronounced when we assayed proliferation and IL-2 abundance in the transduced cells, as reflected by the severe reduction in both proliferation (Fig. 1E) and IL-2 abundance (Fig. 1F) in cells expressing PKC θ ^{HR2A} compared to cells expressing PKC θ ^{WT}. The magnitude of the decrease in IL-2 production in PKC θ ^{HR2A}-expressing T cells was similar whether the cells were stimulated with the CD3 mAb alone or with a combination of both the CD3 and CD28

mAbs (Fig. 1G), implying that signals emanating from the TCR, rather than CD28 costimulation, dictate the effector function of PKC θ (see also fig. S2, B to D). These defects in T cell activation in cells that express PKC θ^{HR2A} were not the result of impaired catalytic activity, because the HR2A mutation did not reduce the catalytic activity of PKC θ in an in vitro kinase assay (Fig. 1, H and I); the kinase-inactive mutant PKC θ^{K409R} showed no activity in the same assay.

Because efficient RV transduction requires prior T cell stimulation, it was possible that PKC θ is required very early during TCR signaling, thereby accounting for the defective activation of preactivated, RV-transduced T cells (Fig. 1). To address this potential concern, we extended our analysis to CD4 $^{+}$ T cells derived from bone marrow (BM) mouse chimeras. These chimeras were generated by reconstituting sublethally irradiated *Rag1* $^{-/-}$ mice with BM cells isolated from *Prkcd* $^{-/-}$ mice and transduced in vitro with PKC θ^{WT} , PKC θ^{HR2A} , or empty vector (EV). Analysis of T cell development in the BM chimeras revealed similar numbers of T cells in the thymus and spleen (fig. S1A), as well as normal distribution and percentages of T cell subsets in the thymus (fig. S1B) and spleen (fig. S1C) of mice reconstituted with PKC θ^{WT} , PKC θ^{HR2A} , or EV. These results are consistent with previous reports that PKC θ is largely dispensable for T cell development (38–40) and, moreover, indicate that pTyr-binding by the PKC θ C2 domain was not required for T cell development.

Despite similar abundances of PKC θ^{WT} and PKC θ^{HR2A} in the transduced peripheral CD4 $^{+}$ T cells from the BM chimeras (fig. S2A), the PKC θ^{HR2A} -transduced BM chimeric CD4 $^{+}$ T cells displayed severe defects in the costimulation-induced increase in CD25 and CD69 abundance (Fig. 2, A and B), IL-2 production (Fig. 2, C and D), and proliferation (Fig. 2E), similar to the in vitro-transduced T cells (Fig. 1). As a control, the non-transduced (GFP $^{-}$) T cells from the same BM chimeras displayed similar amounts of the activation markers CD25 and CD69 (Fig. 2, A and B) and IL-2 (Fig. 2, C and D), independent of whether the mice were transduced with PKC θ^{WT} , PKC θ^{HR2A} , or EV. The modest increase in CD25 and CD69 in EV- and PKC θ^{HR2A} -expressing stimulated T cells (~30%) likely reflects the fact that increases of these activation markers are not entirely and exclusively dependent on PKC θ . We also analyzed the in vitro differentiation of sorted, transduced CD4 $^{+}$ T cells from the same BM chimeras into Th1 versus Th2 cells and found that Th1 differentiation, as assessed by interferon γ (IFN γ) production, of EV- or PKC θ^{HR2A} -expressing T cells was similar to that of PKC θ^{WT} -transduced T cells. In contrast, Th2 differentiation, as assayed by IL-4 production, was strongly reduced in PKC θ^{HR2A} -reconstituted T cells compared to the control PKC θ^{WT} -expressing cells (Fig. 2F and fig. S2F). These results are consistent with studies demonstrating that in vitro Th2, but not Th1, differentiation is critically dependent on PKC θ (6, 7) and, moreover, demonstrate that the pTyr-binding function of PKC θ was required for Th2, but not Th1, differentiation. Of note, CD3 stimulation alone was as effective as combined CD3 plus CD28 costimulation in increasing the abundance of CD25 (fig. S2B) and CD69 (fig. S2C), substantiating the notion that at least the increase of these activation markers by PKC θ is coupled primarily to TCR signals; however, CD28 costimulation did enhance the amount of IL-2 (fig. S2D) and the proliferation rate (Fig. S2E versus Fig. 2E) induced by CD3 stimulation alone.

Taken together, the above results (Figs. 1, 2 and figs. S1, S2) demonstrate that pTyr binding by the PKC θ C2 domain was required for PKC θ -dependent T cell activation, proliferation, and differentiation events but not for T cell development, thereby extending our previous finding that pTyr binding by the C2 domain is also required for NF- κ B activation (36), another well-established PKC θ -dependent function (39–42).

The kinase Zap70 interacts with the C2 domain of PKC θ

To identify a physiologically relevant pTyr-containing protein that associates with PKC θ in stimulated T cells, we fused glutathione S-transferase (GST) to the unmutated (C2^{WT}) or HR2A-mutated (C2^{HR2A}) C2 domain of PKC θ and used these recombinant fusion proteins to pull down candidate proteins from lysates of unstimulated or pervanadate (PV)-stimulated Jurkat E6.1 T cells. PV is a tyrosine phosphatase inhibitor that mimics TCR signals inducing protein tyrosine phosphorylation and, thus, greatly boosts cellular pTyr abundance in T cells. Several pTyr-containing proteins were consistently pulled down by the recombinant C2^{WT} fusion protein, but not by the C2^{HR2A} protein, from lysates of stimulated Jurkat cells (Fig. 3A); these protein bands were absent in lysates of unstimulated cells (Fig. 3C). Among these, protein(s) in the ~60–70-kDa molecular weight range reactive with an anti-pTyr antibody were dominant.

The corresponding Coomassie-stained gel region containing the dominant Tyr-phosphorylated proteins (Fig. 3A) was excised, destained, and subjected to in-gel trypsin digestion followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This analysis revealed eight separate Zap70 peptides that were present in the material pulled down by the PKC θ C2^{WT} domain, but not in pull-downs with C2^{HR2A} (Fig. 3B), potentially identifying Tyr-phosphorylated Zap70 as a ligand of the PKC θ C2 domain. A similar, single ~70-kDa protein band, which was reactive with both Zap70 and pTyr antibodies, was detected in lysates of PV-stimulated primary mouse CD4⁺ T cells following pull-down by GST-C2^{WT} but not by GST-C2^{HR2A} (Fig. 3C, upper panels). Analysis of whole cell lysates (WCL) from the same stimulated cells revealed a range of pTyr-containing proteins, including a band recognized by an antibody against phosphorylated Zap70 (Tyr³¹⁹), indicative of proper T cell stimulation (Fig. 3C, lower panels).

To validate the inducible PKC θ -Zap70 interaction in T cells, we performed a co-immunoprecipitation assay on lysates of B6 (Fig. 3, D and E) and ovalbumin (OVA)-specific OT-II TCR-transgenic (Fig. 3, F and G) CD4⁺ T cells stimulated with superantigen E (SEE)- or OVA-pulsed antigen-presenting cells (APCs), respectively. In both cases, Zap70 co-immunoprecipitated with PKC θ as early as 1 min after stimulation, and the association was still detected after 30 min but declined at 60 min post-stimulation. A much weaker Zap70 signal was observed in unstimulated cells, indicating that the endogenous PKC θ -Zap70 association required TCR or CD28 stimulation, or both, and, hence, Zap70 tyrosine phosphorylation. Collectively, these data identify Zap70 kinase as a PKC θ C2 domain-interacting protein in stimulated T cells.

Phosphorylation of Tyr¹²⁶, Tyr³¹⁵, and Tyr³¹⁹ in Zap70 is critical for binding to PKC θ

To identify the pTyr residue(s) in Zap70 that is (are) required for the binding of phosphorylated Zap70 to the PKC θ C2 domain, we generated five mutants in human Zap70 in a mammalian expression system with phenylalanine (F) replacements of the six Tyr (Y) residues at positions 126, 292, 315, 319, or 492 and 493 together (492/493 double mutant), all of which are known to play various important roles in Zap70 conformation, translocation, and function (43–47). We transiently expressed these mutants, as well as wild-type Zap70 (Zap70^{WT}), in 293T cells, which do not contain endogenous Zap70 or Lck. We stimulated the cells with PV to induce cellular tyrosine phosphorylation, and then used recombinant GST-tagged PKC θ -C2^{WT} protein (or GST protein alone as a negative control) to pull down Zap70 from these lysates. Zap70^{WT}, Zap70^{Y292F}, and Zap70^{Y492/493F} bound to PKC θ -C2^{WT}; in contrast, the Zap70^{Y126F}, Zap70^{Y315F}, and Zap70^{Y319F} did not bind (Fig. 4A). These results imply that Tyr¹²⁶, Tyr³¹⁵, and Tyr³¹⁹ are required for Zap70 to bind to PKC θ .

To determine phosphorylation of the Tyr¹²⁶, Tyr³¹⁵, and/or Tyr³¹⁹ residues was required for Zap70 to directly bind to the PKC θ C2 domain, we used ELISA to assess the binding of immobilized biotin-tagged 12-amino acid synthetic peptides containing each of these three residues (or Tyr²⁹² as a negative control), in both their non-phosphorylated or phosphorylated states, to a soluble recombinant GST fusion protein of the PKC θ ^{WT} C2 domain (PKC θ -C2^{WT}), or GST alone as a negative control. Maximal binding to GST-PKC θ -C2^{WT} (but not GST) was observed with the pTyr¹²⁶ peptide, with substantially lower but detectable binding of the corresponding non-phosphorylated peptide, likely reflecting the contribution of surrounding amino acids to the binding (Fig 4B). This notion is supported by the reduced binding of the pTyr¹²⁶ peptide to GST-PKC θ -C2^{HR2A} compared to GST-PKC θ -C2^{WT} (Fig. 4C). Binding of all other peptides was minor or barely detectable (Fig. 4B). These results strongly implicate pTyr¹²⁶ in directly mediating the binding of Zap70 to the pTyr-binding pocket in the PKC θ C2 domain. As discussed below, the requirement of pTyr³¹⁵ and pTyr³¹⁹ for the Zap70-PKC θ association (Fig. 4A) almost certainly reflects the critical role of these phosphorylated residues in altering the conformation of resting Zap70 to a partially active “open” conformation, which would then likely expose pTyr¹²⁶ for binding to the PKC θ C2 domain.

We next asked whether the kinase Lck was required for the PKC θ -Zap70 association by assessing the ability of recombinant GST-PKC θ -C2^{WT} protein to pull down Zap70 in lysates from PV-stimulated wild-type (JE6.1) and Lck-deficient (Jcam1.6) Jurkat cells. Compared to Zap70 from JE6.1 cells, Zap70 from the Jcam1.6 cells displayed strongly reduced binding to the PKC θ C2 domain (Fig. 4, D and E). The absence of detectable Lck in the C2-bound material (Fig. 4D) rules out the possibility that Lck serves as an intermediate in forming the PKC θ -Zap70 complex, and further supports a direct interaction. These results are consistent with the well-established importance of Lck in initiating TCR signaling by phosphorylating the immunoreceptor Tyr-based activation motifs (ITAMs) of the CD3 complex, thereby enabling recruitment of Zap70 to the TCR-CD3 complex and its subsequent phosphorylation and activation.

pTyr-binding by the PKC θ C2 domain is required for full Zap70 activation and for Zap70-dependent proximal TCR signaling

To further explore the biological relevance of pTyr-binding by PKC θ , we analyzed several TCR- and CD28-proximal early signaling events in T cells from BM chimeric mice transduced with RVs encoding PKC θ^{WT} or PKC θ^{HR2A} . We first analyzed the degradation of the inhibitor of NF- κ B α (I κ B α), and the inducible phosphorylation of the closely related kinases I κ B kinase (IKK) α and IKK β (IKK α/β). These are surrogate markers of the activation of the conventional NF- κ B pathway, which is known to require active PKC θ (39–42). As expected, stimulation of PKC θ^{HR2A} -transduced primary T cells failed to induce IKK α/β phosphorylation or I κ B α degradation (Fig. 5, A and B), confirming the importance of pTyr binding by PKC θ for NF- κ B activation (36).

To analyze the activation of more proximal TCR signaling events, we examined the phosphorylation of Zap70, phospholipase C- γ 1 (PLC γ 1), and the transmembrane adaptor protein linker for activation of T cells (LAT), all of which are activated downstream of TCRs but act upstream of NF- κ B. We found that stimulation of PKC θ^{HR2A} -expressing cells resulted in reduced phosphorylation of Zap70 at Tyr⁴⁹³, which correlates with fully active Zap70 (48), as well as the absence of detectable activating tyrosine phosphorylation of PLC γ 1 and LAT (Fig. 5C and D). In contrast, the overall phosphorylation of Lck and the CD3 subunit TCR ζ was largely intact as determined by a total pTyr antibody. Consistent with impaired PLC γ 1 activation, the activating phosphorylation at Tyr⁵¹¹ on interleukin-2-inducible T-cell kinase (Itk), a tyrosine kinase that phosphorylates and activates PLC γ 1 (49, 50), was also impaired in the PKC θ^{HR2A} -expressing CD4⁺ T cells (Fig. 5E). Similar defects were observed in CD4⁺ T cells from *Prkcg*^{-/-} mice (fig. S3). These findings were unexpected because activating phosphorylations of Zap70, LAT, and PLC γ 1 were traditionally considered to be PKC θ -independent, occurring “upstream” of the activation of PKC θ and other PKC family members (51, 52). These unexpected findings suggest that, contrary to the generally accepted dogma, active PKC θ promotes the activation of these early signal transducers.

PKC θ recruitment to the immunological synapse (IS) and the association with CD28 depend on pTyr binding by the C2 domain

The importance of pTyr-binding by the PKC θ C2 domain for PKC θ function drove us to determine if it is also required for PKC θ localization to the IS. When retrovirally expressed in *Prkcg*^{-/-} OT-II CD4⁺ T cells, GFP-PKC θ^{WT} localized to the IS together with endogenous Zap70 following stimulation with APCs loaded with the OVA peptide (Fig. 6, A and B), consistent with the co-immunoprecipitation data (Fig. 3, D to G). In contrast, GFP-PKC θ^{HR2A} did not translocate to the IS and, instead, remained largely confined to the cytoplasm, with little or no effect on the IS recruitment of Zap70, indicating a largely intact IS formation (Fig. 6, A and B). These results suggest that binding of PKC θ C2 to pTyr was required for the selective IS localization of PKC θ , but not (or only minimally) required for general IS assembly.

We demonstrated previously that inducible association of PKC θ with the cytoplasmic domain of CD28 accounts for the recruitment of PKC θ to the center of the T cell IS and,

moreover, that this association is required for PKC θ -dependent downstream signaling events in TCR-stimulated T cells (31). We wished therefore to find out whether pTyr binding by PKC θ was linked to the stimulation-induced formation of the PKC θ -CD28 complex. Analysis of T cells transduced in vitro with PKC θ^{HR2A} (Fig. 6C) or obtained from PKC θ^{HR2A} -expressing BM chimeras (Fig. 6D) demonstrated that PKC θ^{HR2A} , which does not bind pTyr, was defective in its ability to associate with CD28 following T cell stimulation. This result suggests that pTyr binding by PKC θ is required for formation of the PKC θ -CD28 complex. This notion is supported by the finding that a PKC θ mutant that does not bind to CD28 (V36), in which the native CD28-binding V3 domain has been replaced with the corresponding domain of PKC δ (31), was still capable of efficiently associating with Zap70 after stimulation with SEE-pulsed APCs (Fig. 6E). Thus, the association of PKC θ with Zap70 appears to be largely independent of its association with CD28. We also found that, in contrast of PKC θ^{WT} expression, expression of PKC θ^{HR2A} did not alter the stimulation-induced recruitment of Zap70 to TCR ζ (fig. S4A), suggesting that the binding of Zap70 to PKC θ does not exclude its recruitment to TCR ζ . It is possible that two distinct pools of Zap70 associate with PKC θ and with TCR ζ . However, PKC θ^{HR2A} , which did not associate with Zap70 (fig. S4B), was also impaired in its ability to co-immunoprecipitate with the adaptor protein CARMA1, a critical component of the TCR-induced NF- κ B signaling pathway (53), in stimulated CD4 $^{+}$ T cells (fig. S4C), consistent with the other defective steps in this pathway in PKC θ^{HR2A} -expressing cells (Fig. 5, A and B).

DISCUSSION

PKC θ , a critical enzyme in T cell biology, is subject to complex regulation at several levels (2, 3). Recently we revealed that the N-terminal C2 domain of PKC θ is a pTyr-binding domain (36). As initial evidence for the importance of pTyr-binding by PKC θ , we also showed that a PKC θ mutant incapable of binding pTyr was deficient in its ability to activate an NF- κ B reporter gene in Jurkat T cells (36). However, our previous study did not reveal the physiological pTyr-containing ligand that binds to PKC θ in T cells or whether this newly discovered property of the C2 domain is required for downstream effector functions of PKC θ , including proliferation, cytokine expression, and Th differentiation of primary T cells. Here we demonstrated that PKC θ^{HR2A} , a mutant that cannot bind pTyr, was unable to restore TCR-induced T cell activation in vitro or in vivo, as indicated by defective activation marker induction (CD25 and CD69), proliferation, IL-2 production, and Th2 differentiation, events all known to be PKC θ -dependent. In contrast, the in vitro Th1 differentiation of PKC θ^{HR2A} -expressing T cells remained intact, consistent with findings that Th1 differentiation is largely PKC θ -independent (6, 7). The HR2A mutation did not affect thymic T cell development, in agreement with the minor role of PKC θ in T cell development (38–40). This lack of role for PKC θ in T cell development is likely explained by redundancy with another PKC family member, PKC η (38). This finding also implies that the critical role of Zap70 kinase, the PKC θ -binding protein we identified here, in T cell development (54, 55), is independent of its interaction with PKC θ .

Using tandem MS, we identified phosphorylated Zap70 kinase as a ligand that binds to the PKC θ C2 domain in TCR-stimulated T cells and confirmed this interaction by co-immunoprecipitation of a PKC θ -Zap70 complex from stimulated T cells, including antigen

(OVA)-stimulated primary OT-II CD4⁺ T cells. This interaction was further confirmed by the prominent IS colocalization of PKC θ ^{WT} with Zap70 in stimulated T cells, which was substantially reduced in cells expressing the pTyr-non-binding PKC θ ^{HR2A} mutant. PKC θ , which is known to associate with CD28 in PMA- or antigen-stimulated CD4⁺ T cells (5, 31), is likely to have an opportunity to physically interact with Zap70 in TCR microclusters that are formed in the periphery of the IS early after T cell stimulation because CD28 has been shown to reside in these early Zap70-containing TCR microclusters and to segregate later, together with PKC θ , from these TCR microclusters, localizing in an outer subregion of the cSMAC (5). Although we concentrated here on characterizing the PKC θ -Zap70 interaction, it is worth noting that the recombinant unmodified C2 domain (but not the HR2A-mutated C2 domain) pulled down a few unidentified pTyr-containing proteins in addition to Zap70. Thus, PKC θ may well interact with other tyrosine-phosphorylated proteins, the identity as well as importance of which remain to be established, in T cells. However, the biological relevance of the PKC θ -Zap70 interaction is supported by, and likely provides an explanation for, earlier findings that Zap70 promotes PKC θ activation (56) and that Zap70 is required for recruitment of PKC θ to the T cell IS (57).

Using a peptide-binding ELISA, we identified Zap70 Tyr¹²⁶ as a highly likely site that can, when phosphorylated, mediate direct association of Zap70 with PKC θ . In contrast, peptides corresponding to pTyr³¹⁵ or pTyr³¹⁹ in Zap70 did not bind the PKC θ C2 domain in this assay, although mutations of these two residues to phenylalanine abolished the interaction of the mutant Zap70 proteins with the C2 domain of PKC θ in pull-down assays. This apparent discrepancy is most likely attributed to the well-established role of pTyr³¹⁵ and pTyr³¹⁹ in regulating the conformation and, consequently, the activity of Zap70. These two residues are important components of an autoinhibitory switch that controls the activation of Zap70. According to one model, in their unphosphorylated resting state, mimicked by mutation of these residues to phenylalanine, these residues keep the kinase in a closed, inactive state (43, 44, 48). Subsequent Lck-mediated phosphorylation or trans-autophosphorylation of these Tyr residues in TCR-bound and partially active Zap70 results in a conformational change that exposes its kinase domain, allowing trans-autophosphorylation of Tyr^{492/493} and other Tyr residues (such as Tyr¹²⁶) and resulting in a fully active kinase (46, 48, 58). A more recent study revealed that stabilizing the open conformation of Zap70 through phosphorylation of Tyr^{315/319} by Lck or trans-autophosphorylation prolongs the dwell time of Zap70 at the TCR and, thereby, increases the probability of its activation through Tyr⁴⁹³ phosphorylation (45). On the other hand, trans-autophosphorylation of Tyr¹²⁶ releases active Zap70 molecules from the TCR into the plasma membrane, thereby promoting signal amplification and propagation (47). Hence, a likely scenario is that, following TCR stimulation of resting T cells and Lck-mediated ITAM phosphorylation, Zap70 Tyr^{315/319} phosphorylation or TCR binding induces a conformational change that exposes Tyr¹²⁶ for phosphorylation and direct binding to the C2 domain of PKC θ . This could potentially promote Zap70 release from TCR complex and allow it to efficiently phosphorylate its downstream target, LAT, and subsequently activate LAT-associated signaling proteins such as PLC γ 1 and Itk (59, 60). Consistent with such a scenario, the Y126F mutation of Zap70, which would prevent its binding to the C2 domain of PKC θ , is associated with reduced LAT phosphorylation and Ca²⁺ signaling (47), similar to the reduced phosphorylation of LAT,

PLC γ 1 and Itk, which we observed here in T cells expressing the HR2A-mutated PKC θ , which cannot bind and recruit Zap70.

The association between PKC θ and Zap70 could potentially serve another purpose: facilitating integration of distinct signaling modules initiated by TCR and CD28 ligation. The TCR, which recruits Zap70, and CD28, which recruits PKC θ , have been shown to co-localize to peripheral TCR microclusters during the early (~2 min) stage of IS assembly, and to later (~20 min) segregate into distinct subdomains in the mature IS (5). Thus, the inducible PKC θ -Zap70 association might be facilitated by the colocalization of the TCR and CD28 in the same early peripheral microclusters, thereby facilitating the integration of distinct signaling modules that promote the activation of NF- κ B (61) and other downstream effectors.

Unexpectedly, PKC θ -deficient T cells reconstituted with the PKC θ^{HR2A} mutant displayed substantial defects in early TCR signaling events, such as the activating phosphorylation of LAT, PLC γ 1, and Itk. LAT is a direct Zap70 substrate, and it is required for the recruitment and activation of PLC γ 1 and Itk. Thus, the impaired Zap70-dependent phosphorylation of LAT in the presence of PKC θ^{HR2A} provides additional, indirect support to our conclusion that full Zap70 activation or, rather, its increased ability to promote highly efficient downstream signaling due to its release from the TCR (47), is mediated by its interaction with PKC θ . More proximal TCR signaling events such as the tyrosine phosphorylation of Lck and TCR ζ were unaffected in the PKC θ^{HR2A} -expressing cells. We documented similar defects in early TCR signaling in primary *Prkcd*^{-/-} CD4⁺ T cells. These defects were surprising because the activation of these proximal signal transducers is traditionally considered to occur upstream or independently of PKC activation (51, 52). However, in retrospect, our findings are consistent with and potentially explain previous reports that PKC θ controls the activation of PLC γ 1, LAT, and Itk, and that *Prkcd*^{-/-} CD4⁺ T cells display defects in TCR-induced Ca²⁺ signaling and in Ca²⁺-dependent activation of nuclear factor of activated T cells (NFAT), a transcription factor necessary for T cell activation (39, 62, 63). Thus, rather than functioning in a linear TCR signaling scheme strictly downstream of Zap70, LAT, and PLC γ 1, PKC θ likely functions in a complex feedback loop(s) triggered by TCR and CD28 stimulation, with substantial cross-regulation among components of these loops, including regulation of TCR-proximal signaling such as the activation of Zap70, LAT, and PLC γ 1.

The presence of Zap70 specifically in T cells and its critical role in controlling most, if not all, TCR signaling events (43), including PKC θ function as demonstrated here, implicate this tyrosine kinase as a potentially attractive drug target in pathological conditions wherein suppression of T cell hyperactivity is desired, such as autoimmunity and transplant rejection. However, success in developing selective Zap70 catalytic inhibitors for clinical use has been limited. Our findings that pTyr binding to PKC θ is important for intact TCR signaling and effector functions and that PKC θ is required for optimal Zap70 activation raise the possibility that allosteric inhibitors that prevent binding of pTyr-containing proteins to PKC θ , which is also abundant in T cells, could represent an attractive alternative strategy for selective T cell immunosuppression. More studies are required to further explore the

molecular details of the interaction and cross-regulation between PKC θ and Zap70, and to validate the potential utility of this interaction as a drug target.

MATERIALS AND METHODS

Mice, primary cells and cell lines

C57BL/6 (B6), OT-II TCR-transgenic, *Rag1*^{-/-}, and *Prkcd*^{-/-} mice (a gift from D. Littman) were maintained under specific pathogen-free conditions and manipulated according to a protocol approved by the La Jolla Institute for Immunology Animal Care Committee. CD4⁺ T cells were isolated by anti-mouse CD4 magnetic particles (L3T4; BD IMag) and cultured in RPMI-1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 1 mM MEM nonessential amino acids, and 100 U/ml each of penicillin G and streptomycin (Life Technologies).

Jurkat E6.1 cells or the Lck-deficient Jcam1.6 T cells were maintained in complete RPMI-1640 medium supplemented with 10% FBS, penicillin, and streptomycin. HEK293T cells and Platinum-E packaging cells were cultured in complete DMEM (Gibco) containing penicillin, streptomycin, and 10% (v/v) FBS. All cell lines were obtained from the American Type Culture Collection (ATCC).

Plasmids

The cDNA for full-length human PKC θ was generated by PCR amplification and subcloned into the bicistronic IRES retroviral vector (RV) pMIG (64) for generation of bone marrow (BM) chimeras, or into the C-terminal eGFP-encoding fusion vector pMX for *in vitro* transduction of cells. A cDNA encoding the C2 domain of human PKC θ (aa 1–144) was subcloned into pGEX-4T2 to express PKC θ -C2 downstream of an N-terminal GST fusion tag. PKC θ -V36 (replacement of PKC θ -V3 with amino acids 282–358 of PKC δ) was constructed by overlapping PCR (31). Full-length human Zap70 in the mammalian expression vector pEFneo was described previously (65). Point mutants were generated with the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions.

RV transduction and stimulation

Platinum-E packaging cells were plated in a 6-well plate in 2 ml RPMI-1640 medium plus 10% FBS. After 24 hours, cells were transfected with empty pMIG or pMX vector or the appropriate PKC θ -expressing vectors (3 μ g) with TransIT-LT1 transfection reagent (Mirus Bio). After overnight incubation, the medium was replaced, and cultures were maintained for another 24 hours. Culture supernatants were then collected and filtered, supplemented with polybrene (8 μ g/ml), and used to transduce CD4⁺ T cells that had been preactivated for 24 hours on plates coated with mAbs recognizing CD3 and CD28 (2 μ g/ml each). After centrifuging plates for 1.5–2 hours at 2,000 rpm, supernatants were replaced by fresh FBS-containing RPMI-1640 plus recombinant IL-2 (100 U/ml; Biolegend). Cells were incubated for another 24 hours at 37°C. On day 3, cells were washed, transferred to new plates, and cultured in RPMI-1640 medium containing FBS and IL-2 without stimulation for 2 additional days before restimulation with mAbs recognizing CD3 or CD28 or both. For Th

cell differentiation, CD4⁺ T cells transduced as described above were cultured under polarizing conditions (Th1: 100 U/ml IL-2, 20 U/ml IL-12 and 10 µg/ml IL-4 mAb; Th2: 100 U/ml IL-2, 200 U/ml IL-4, 10 µg/ml each IL-12 mAb and IFNγ mAb). After resting for 3 days, cells were restimulated for 8 hours on plates coated with CD3 and CD28 mAbs (1 µg/ml each) without additional cytokines, and intracellular cytokine staining (ICS) was performed in the presence of GolgiStop (BD Biosciences).

BM chimeras

BM cells were flushed from the femurs and tibias of *Prkcd^{-/-}* mice, subjected to a lineage-negative selection with a lineage cell depletion kit (Miltenyi Biotech), and cultured for 24 hours in DMEM (Gibco) supplemented with FBS (10% v/v), IL-3 (10 ng/ml), IL-6 (20 ng/ml), and SCF (100 ng/ml; all from PeproTech) before RV transduction for 2 consecutive days as described above. RV-transduced BM cells were injected intravenously into sublethally irradiated (500 rad) *Rag1^{-/-}* mice 2 days later. Recipient mice were euthanized and analyzed ~8 weeks after reconstitution.

Flow cytometry and intracellular staining

Cell proliferation was assessed by flow cytometry of T cells prelabeled with CellTrace-Violet (CTV) Cell Proliferation dye (Life Technologies), and measured by CTV dilution after stimulation for 72 hours. Abundances of CD25 and CD69 were determined by live cell surface staining on ice with mAbs recognizing these activation markers (diluted 1:200 in 1% BSA/PBS). For intracellular staining, cells were fixed and permeabilized with Cytotfix/Cytoperm buffer (BD Biosciences), followed by staining with antibodies recognizing phosphorylated Itk or cytokines diluted 1:200 in Perm/Wash buffer (BD Biosciences). For PKCθ, fixed and permeabilized cells were stained with mouse primary antibodies against PKCθ (BD Biosciences) followed by an Alexa Fluor 647-conjugated anti-mouse secondary antibody. Acquisition was performed on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (v10; FlowJo LLC).

Immunofluorescence microscopy

APCs were prepared from wild-type B6 mice by depleting splenocytes of CD4⁺ T cells using beads coated with a CD4 mAb (L3T4). APCs were stained with CellTracker Blue (CMAC; 20 µM) and then pulsed for 30 min at 37°C with 5 µM OVA peptide. Cells were washed twice in PBS and mixed with T cells at a ratio of 1:1, followed by incubation for 20 min at 37°C. T cell-APC conjugates were plated on poly-L-lysine-coated slides and incubated for 15 min at room temperature before fixation with 4% (w/v) paraformaldehyde and permeabilization with PBS containing BSA (1% w/v) and Triton X-100 (0.2% v/v). Nonspecific binding was blocked by incubation for 30 min with PBS/2% BSA, and cells were stained overnight with a rabbit Zap70 primary antibody at 4°C. Samples were washed three times in PBS/Tween20 and incubated for 1 hour at room temperature with Alexa Fluor 555-coupled goat anti-rabbit secondary antibody (Invitrogen). Images were captured by a Leica SP5 laser scanning confocal microscope equipped with a 63x objective lens.

Immunoprecipitation, immunoblotting, and GST pull-down assays

Cells were lysed in 1% digitonin (w/v; for CD28 co-immunoprecipitations) or 1% NP-40 (v/v; for all other immunoprecipitations and pull-downs) in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) supplemented with protease inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM PMSF) and phosphatase inhibitors (5 mM NaPPi and 1 mM Na₃VO₄). Cells were incubated for 2 hours with the antibody, and proteins were immunoprecipitated overnight at 4°C with protein G–Sepharose beads (GE Healthcare). Immunoprecipitated proteins were resolved by SDS–PAGE, transferred onto a PVDF membrane, and probed overnight at 4°C with primary antibodies, followed by incubation for 1 hour at room temperature with HRP-conjugated secondary antibodies. Signals were visualized by enhanced chemiluminescence (ECL; GE Healthcare) and exposed to X-ray film. Densitometry analysis was performed with ImageJ software. GST or GST fusion proteins were batch expressed in *E. coli* BL21 cells, purified, and dialyzed overnight against PBS/10% glycerol. GST pull-down assays were performed as previously described (66).

ELISA

Aliquots of transduced *Prkcg*^{-/-} CD4⁺ T cells (5×10^4) were stimulated for 48 hours with CD3 or CD28 mAbs, or both, and IL-2 concentration in culture supernatants was determined by ELISA according to the manufacturer's instructions (Biolegend). A 96-well plate (Corning Costar) was coated overnight at 4°C with an IL-2 mAb. Triplicates of IL-2 standards and supernatants from cultured cells were then added to the plate, followed by a 2-hour incubation at room temperature. A biotinylated IL-2 antibody was added to the plate, followed by incubation for 1 hour, and then streptavidin-HRP was added, followed by incubation for 30 min, all at room temperature. After washing, the amount of bound avidin was assessed with TMB peroxidase that was acidified by 2 N H₂SO₄. Absorbance at 450 nm was measured in a SpectraMax plate reader (Molecular Devices).

For peptide ELISA, the following N-terminal biotinylated peptides were synthesized and purified to >90% as judged by HPLC and mass spectrometry (GenScript): Y126, AMVRDYVRQ**T**WK; pY126, AMVRDpYVRQ**T**WK; Y292, LNSDGYT**P**EPAR; pY292, LNSDGpYT**P**EPAR; Y315/319, TSVYESPYSD**P**E; pY315, TSVpYESPYSD**P**E; pY319, TSVYESPpYSD**P**E; 2pY (pY315 and pY319), TSVpYESPpYSD**P**E. Biotinylated peptides (10 µg/ml in 100 µl PBS) were added to microtiter wells of a streptavidin-precoated plate (Pierce) and incubated overnight at 4°C. Proper and equal binding of the different peptides to the wells was confirmed by adding HRP-streptavidin to a duplicate set of wells. Non-specific binding sites were blocked by incubation with 300 µl of 5% BSA/PBS for 1 hour at room temperature. Following washing with PBST (0.5% Tween-20/PBS), GST, or GST-C2 proteins [1 µg in 1% (w/v) NP-40 lysis buffer] were added for 1 hour. After further washing, the wells were incubated for an additional hour with a mouse antibody against GST (1:2000 in PBST), followed by 1 hour incubation with HRP-conjugated anti-mouse Ig at room temperature. Samples were analyzed in triplicate in both peptide-coated and uncoated control wells, and ELISA readout was performed as described above.

PKC θ *in vitro* kinase assay

HEK293T cells transfected with empty vector or expression plasmids encoding Myc-tagged PKC θ^{WT} , PKC θ^{HR2A} , or kinase-dead PKC θ^{K409R} , were lysed and immunoprecipitated with an antibody against Myc. Immunoprecipitates were washed twice with lysis buffer and twice with PKC θ kinase buffer (20 mM HEPES, 10 mM MgCl₂ and 0.1 mM EGTA), then resuspended in 25 μ l of the corresponding kinase buffer containing 5 μ Ci [γ -³²P] ATP (Perkin Elmer), 20 μ M ATP, and 1 μ g of myelin basic protein (MBP) substrate (Sigma), in the presence or absence of cofactors, phosphatidylserine (200 μ g/ml; Promega) and 10 μ M PMA. Samples were incubated for 30 min at 30°C with gentle shaking. The reaction was stopped by the addition of 5x loading buffer. Samples were incubated for 10 min at 95°C, and separated by SDS-PAGE. Gels were exposed to a BAS Storage Phosphor screen (GE Healthcare) and scanned with the Typhoon Trio Imager (GE Healthcare).

LC-MS/MS

Protein bands were excised from Coomassie-stained gels and destained, then subjected to overnight in-gel trypsin digestion [12.5 ng/ μ l in 50 mM (NH₄)HCO₃ at 37°C]. The resulting peptides were extracted with 50% acetonitrile/5% formic acid and dried in a vacuum centrifuge. Dried peptides were dissolved in 0.1% formic acid and analyzed by online nanoflow LC-MS/MS on an Agilent 1200 quaternary HPLC system (Agilent) connected to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) through an in-house built nanoelectrospray ion source. Peptide mixtures were pressure-loaded onto a capillary column (75- μ m internal diameter) packed with 15-cm of 3- μ m Aqua C18 resins (Phenomenex). Peptides were separated using a 60-min gradient from 5% to 60% acetonitrile in 0.1% formic acid at a flow rate of 300 nL/min (through split). As peptides were eluted from the analytical column, they were electrosprayed (distal 2.5 kV spray voltage) into the mass spectrometer. The MS method consisted of an FT full-scan MS analysis (400–1600 m/z, 60000 resolution) followed by data-dependent MS/MS scans of the 10 most intense precursors at a 35% normalized collision energy with dynamic exclusion for 60 seconds. Application of mass spectrometer scan functions and HPLC solvent gradients was controlled by the Xcalibur data system (Thermo Fisher Scientific). MS/MS spectra were extracted using RawXtract (version 1.9.9) (67) and searched with the ProLuCID algorithm (68) against a human UniProt database concatenated to a decoy database, in which the sequence for each entry in the original database was reversed (69). ProLuCID search results were assembled and filtered using the DTASelect (version 2.0) algorithm (70) with a 10 ppm delta mass cutoff of the peptide masses and a false positive rate below 1%. The LC-MS/MS data have been deposited in the UCSD MassIVE database, <https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp>, with accession number MSV000083562.

Antibodies and other reagents

The antibodies and some of the chemical reagents used in this study and their working concentrations or dilutions are listed in table S1.

Statistical analysis

Data for all experiments were analyzed with Prism software (GraphPad). Multiple *t*-tests were used for comparison among experimental groups. *P* values of less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Deficient activation of T cells from PKC θ knockout mice expressing the pTyr non-binding PKC θ mutant HR2A.

(A) Alignment of sequences surrounding the pTyr-binding site in the C2 domains of nPKC subfamily members. Residues that form the pTyr-binding pocket are in gray, and Ala mutations of His⁶³ and Arg⁶⁸ in the HR2A mutant are shown.

(B and C) Immunoblot for PKC θ in sorted (GFP⁺) primary CD4⁺ T cells that were transduced with control retrovirus (RV) encoding GFP alone (vector) or GFP and either PKC θ ^{WT} or PKC θ ^{HR2A}. Actin is a loading control. Results representative of 5 independent

experiments are shown in **(B)**. Quantified band intensity ratio values **(C)** are means $\pm$ SEM pooled from 5 independent experiments.

(D–F) Flow cytometry analysis of the surface abundance of the T cell activation markers CD25 and CD69 **(D)**, proliferation **(E)**, or IL-2 abundance **(F)** of CD4⁺ T cells that were either left unstimulated (No Stim) or restimulated with mAbs specific for CD3 and CD28 (α -CD3+CD28). Numbers above bracketed lines indicate % positive **(D and F)** or % proliferating **(E)** cells. Mean fluorescence intensities (MFI) of CD25 and CD69 are shown. Representative plots from 5 independent experiments are shown.

(G) Abundance of IL-2 in supernatants of sorted CD4⁺ T cells transduced with RV encoding PKC θ^{WT} , PKC θ^{HR2A} and left unstimulated (0), restimulated with CD3 only (α -CD3) or CD3 and CD28 mAbs (α -CD3+CD28). Data are means $\pm$ SEM of 9 biological replicates pooled from 3 independent experiments. **(H and I)**. *In vitro* kinase assay with Myc-tagged PKC θ^{WT} , PKC θ^{HR2A} , or the kinase-inactive mutant PKC θ^{K409R} immunoprecipitated (IP) from transfected HEK293T cells. Immunoprecipitated proteins were mixed with radiolabeled ATP and myelin basic protein (MBP), and the reactions were separated on a gel for autoradiography and for immunoblotting for Myc and MBP. **(H)** is the representative blot, and **(I)** are band densitometry ratio (means $\pm$ SEM) pooled from 3 independent experiments. ns, not significant, * $P < 0.05$, ** $P < 0.01$.

Fig. 2. Deficient activation of CD4⁺ T cells from BM chimeric mice reconstituted with PKCθ knockout BM cells expressing PKCθ^{HR2A}.

(A–D) Flow cytometry analysis of the surface abundance of CD25 and CD69 (A and B) and the production of IL-2 (C and D) by transduced (GFP⁺) or non-transduced (GFP⁻) naïve CD4⁺ T cells from BM chimeric mice reconstituted with PKCθ knockout BM cells expressing empty vector (vector), PKCθ^{WT}, or PKCθ^{HR2A}. Cells were left unstimulated or restimulated with CD3 and CD28 mAbs. Numbers above bracketed lines (A) and in the gated boxes (C) indicate % positive cells from a single representative mouse in each group. MFI values of CD25 and CD69 (A) or IL-2 (C) are shown, and averaged data pooled from

six mice/group are shown in **(B)** and **(D)**. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Results are representative of 4 independent experiments.

(E and F) Flow cytometry analysis of the proliferation **(E)** and the amounts of IFN γ (Th1) or IL-4 (Th2) cytokines **(F)** in sorted (GFP $^{+}$) CD4 $^{+}$ cells as in **(A to D)**. Numbers in **(E)** and **(F)** indicate the % of proliferating cells or the frequency of IFN γ - or IL-4-containing cells among gated CD4 $^{+}$ cells, respectively. Results are representative of 6 mice/group and 3 independent experiments.

Fig. 3. The PKCθ C2 domain interacts with the kinase Zap70.

(A) Purified recombinant GST-tagged WT or HR2A mutant forms of the PKCθ C2 domain, GST-C2 and GST-C2-HR2A, respectively, were used for pull-down assays of lysates from unstimulated or PV-stimulated Jurkat T cells. Bound material was separated by SDS-PAGE and immunoblotted for phosphotyrosine (pTyr) and GST. The boxed area was subjected to LC-MS/MS analysis. Data are representative of 3 independent experiments.

(B) Identity of some of the peptides resolved by LC-MS/MS from PV-stimulated Jurkat T cells.

(C) Representative pull-down experiment using purified recombinant GST-C2 and GST-C2-HR2A to pull down proteins from lysates of unstimulated or PV-stimulated primary CD4⁺ T cells from PKC θ knockout mice. Pulled-down material (top panels) or whole-cell lysates (WCL) were analyzed by immunoblotting for the indicated proteins. Data are representative of 3 independent experiments.

(D–G) Primary B6 (D and E) or OT-II (F and G) CD4⁺ T cells were left unstimulated or stimulated for the indicated times with BM-derived dendritic cells (APC) pulsed with SEE or OVA peptide, respectively. PKC θ immunoprecipitates (IP) or WCL were immunoblotted for the indicated proteins. Representative immunoblots of 3 independent experiments are shown (D and F) alongside quantification of Zap70 by densitometry (E and G) pooled from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$.

Fig. 4. Identification and characterization of Zap70 pTyr residues required for binding to the PKC θ C2 domain.

(A) Purified recombinant GST (control) or GST-C2^{WT} was used to pull down proteins in lysates of PV-stimulated 293T cells expressing WT Zap70 or the indicated Zap70 mutants. Pulled-down material and whole-cell lysates (WCL) were separated by SDS-PAGE, stained with Ponceau S, and immunoblotted for Zap70. This experiment is representative of 3 independent experiments.

(B) Binding of recombinant GST and GST-C2^{WT} to the indicated immobilized synthetic Zap70 peptides was assessed by an ELISA. Non, non-peptide-coated control wells.

(C) Binding of the indicated recombinant proteins to wells coated with the pTyr¹²⁶ peptide determined by ELISA. Pooled data from 4 independent experiments are shown in (B) and (C), ** $P < 0.01$.

(D and E) GST pull-down with lysates of WT (JE6.1) or Lck-deficient (Jcam1.6) Jurkat cells that were left unstimulated or stimulated with PV or CD3 and CD28 mAbs as indicated. C2^{WT}-bound proteins or WCL were immunoblotted for the indicated proteins. Data are representative of 3 independent experiments. Pooled data from 3 independent experiments as (D) were quantitated by densitometry and shown in (E). ** $P < 0.01$, *** $P < 0.001$.

Fig. 5. Binding of pTyr by PKCθ is required for early TCR signaling.

(A to D) GFP⁺ primary CD4⁺ T cells were isolated from BM chimeric mice reconstituted with PKCθ knockout BM cells transduced with RV encoding GFP and either PKCθ^{WT} or PKCθ^{HR2A} (A to C) or empty vector (C). The cells were left unstimulated or stimulated with mAbs against CD3 and CD28 for 5 min before cell lysates were subjected to SDS-PAGE and immunoblotted for the indicated proteins. p-Lck and p-TCRζ were determined by the dominant lanes of molecular weight around 55 kDa (Lck) and 23 kDa (TCRζ) from the total pTyr blot (4G10), respectively. Representative raw data are shown in (A) and (C) and

pooled data from 3 independent experiments were quantitated by densitometry in **(B)** and **(D)**. ns, not significant, * $P < 0.05$, ** $P < 0.01$.

(E) Cells isolated and stimulated as in **(A)** and **(C)** were fixed and stained intracellularly with an APC-conjugated antibody against phosphorylated Itk (p-Itk). Plot is representative of 3 independent experiments.

Fig. 6. pTyr binding by PKCθ is required for PKCθ IS localization and association with CD28. (A) Confocal imaging of *Prkcd*^{-/-} OT-II CD4⁺ T cells that were transduced with an RV encoding GFP-tagged WT or HR2A mutant PKCθ (green), mixed with APCs labeled with the cell-tracking dye CMAC (blue), and pulsed with OVA peptide (+OVA). Fixed conjugates were stained for Zap70 plus a secondary fluorescent antibody (AF555). A representative cell is shown. Scale bar, 10 μm. (B) Quantification of PKCθ localization within (IS) or outside of (No IS) the IS in the T cell-APC conjugates analyzed in (A). Analysis was performed only on conjugates that displayed IS-localized Zap70 and detectable PKCθ. *n* = ~120. (C) Co-immunoprecipitation of PKCθ and CD28. (D) PKCθ association with CD28 in a rescue experiment. (E) The δV3 mutant of PKCθ fails to associate with Zap70 and CD28.

(**C** to **E**) GFP⁺ CD4⁺ T cells transduced *in vitro* (**C** and **D**) or derived from BM chimeras reconstituted *in vivo* (**E**) with pMIG vectors expressing PKC θ ^{WT}, PKC θ ^{HR2A}, or PKC θ - δ V3 were stimulated with mAbs against CD3 and CD28 for 5 min. CD28 and PKC θ interaction was detected by co-immunoprecipitation (co-IP). Representative blots are shown above the corresponding band densitometry quantifications pooled from 3 independent experiments. ns, not significant, ** $P < 0.01$, *** $P < 0.001$.