The EMBO Journal (2005) 24, 3869–3880 | © 2005 European Molecular Biology Organization | All Rights Reserved 0261-4189/05
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THE EMBO JOURNAL

Critical role of novel Thr-219 autophosphorylation for the cellular function of PKCθ in T lymphocytes

Nikolaus Thuille[1,4], Isabelle Heit[2,4], Friedrich Fresser[1], Nina Krumböck[1], Birgit Bauer[1], Sabine Leuthaeusser[2], Sascha Dammeier[2], Caroline Graham[3], Terry D Copeland[3], Steve Shaw[3] and Gottfried Baier[1,*]

[1]Department for Medical Genetics, Molecular and Clinical Pharmacology, Innsbruck Medical University, Innsbruck, Austria; [2]ALTANA Pharma AG, Konstanz, Germany and [3]Experimental Immunology Branch, National Cancer Institute, Bethesda, MD, USA

Phosphopeptide mapping identified a major autophosphorylation site, phospho (p)Thr-219, between the tandem C1 domains of the regulatory fragment in protein kinase C (PKC)θ. Confirmation of this identification was derived using (p)Thr-219 antisera that reacted with endogenous PKCθ in primary CD3$^+$ T cells after stimulation with phorbol ester, anti-CD3 or vanadate. The T219A mutation abrogated the capacity of PKCθ to mediate NF-κB, NF-AT and interleukin-2 promoter transactivation, and reduced PKCθ's ability in Jurkat T cells to phosphorylate endogenous cellular substrates. In particular, the T219A mutation impaired crosstalk of PKCθ with Akt/PKBα in NF-κB activation. Yet, this novel (p)Thr-219 site did not affect catalytic activity or second-messenger lipid-binding activity *in vitro*. Instead, the T219A mutation prevented proper recruitment of PKCθ in activated T cells. The PKCθT219A mutant defects were largely rescued by addition of a myristoylation signal to force its proper membrane localization. We conclude that autophosphorylation of PKCθ at Thr-219 plays an important role in the correct targeting and cellular function of PKCθ upon antigen receptor ligation.

The EMBO Journal (2005) **24,** 3869–3880. doi:10.1038/sj.emboj.7600856; Published online 27 October 2005
Subject Categories: signal transduction; immunology
Keywords: autophosphorylation; cellular function; IL-2 activation; PKCθ; T cells

Introduction

Protein kinase C (PKC)θ, a member of the AGC protein serine/threonine kinases, plays a critical role in T-cell-antigen-receptor (TCR) signaling (Baier, 2003; Tan and Parker, 2003). Following T-cell stimulation, PKCθ translocates to the center of the immunological synapse, where it colocalizes with the TCR. The loss of PKCθ function causes immunosuppression, characterized by mostly TCR-unresponsive T cells. Mice lacking PKCθ were mainly deficient in the production of interleukin-2 (IL-2) due to impaired NF-κB, AP-1 and NF-AT transactivation (Sun *et al*, 2000; Pfeifhofer *et al*, 2003). The absence of PKCθ leads to the induction of T-cell anergy (Berg-Brown *et al*, 2004). Additionally, PKCθ was required for the development of a robust immune response controlled by Th2 cells (Marsland *et al*, 2004).

Regulation of nearly all AGC-protein kinases requires transphosphorylation of serine/threonine residues in the activation loop and autophosphorylation of the turn and hydrophobic motif (for reviews, see Newton, 2003; Parker and Murray-Rust, 2004). Conventional PKC isotypes are first phosphorylated in the activation loop by an upstream kinase, PDK-1 or a related enzyme, a step that is essential for its catalytic activity. Then, turn and hydrophobic motif sites are autophosphorylated, and cPKC isotypes adopt a mature and stable conformation ready to be activated by diacylglycerol (DAG) or phorbol ester. For instance, PKCβ has activation loop-, turn- and hydrophobic motif sites at Thr-500, Ser-641 and Ser-660, respectively, and these sites are substantially phosphorylated *in vivo* (Dutil *et al*, 1998). Nevertheless, the regulation of the PKC phosphorylation status is still not fully understood and may well differ for the different PKC subfamily members; in T cells, these three classical phosphorylation sites of PKCθ (in the activation loop, turn motif and hydrophobic motif) are constitutively phosphorylated and are only slightly induced upon receptor ligation (Liu *et al*, 2002, Freeley *et al*, 2005; and G Baier, unpublished). However, others have reported that the activation loop is inducibly phosphorylated by PDK1 (Lee *et al*, 2005). In addition, PKCθ may be tyrosine phosphorylated (Bi *et al*, 2001). In addition, at least one activation-induced autophosphorylation site appears to exist outside the catalytic domain in PKCθ (Bauer *et al*, 2001), but this site has not been identified nor studied functionally.

We have investigated regulatory autophosphorylation events within the PKCθ molecule, which may affect its isotype-selective function in T cells. Here we report a predominant autophosphorylation site, Thr-219, a site within the hinge region of the tandem C1 domains of the regulatory fragment of PKCθ. Thr-219 is phosphorylated by isolated recombinant PKCθ kinase *in vitro* and its occupancy occurs at significant stoichiometry in intact cells. *In vitro*, a neutral exchange mutation of Thr-219, T219A, does not impair second-messenger lipid binding and, in contrast to the established autophosphorylation sites within the catalytic domain of PKCθ (Liu *et al*, 2002), does not affect the catalytic activity of PKCθ. Nevertheless, our data indicate that phosphorylation of Thr-219 regulates targeting and cellular function of PKCθ, suggesting that phosphorylation at Thr-219 of PKCθ plays a role in its interaction with protein partners and/or effectors. Indeed, Thr-219 phosphorylation of PKCθ is required to achieve the established physical and functional crosstalk of

*Corresponding author. Section for Human Genetics, Innsbruck Medical University, Schoepfstraβe 41, 6020 Innsbruck, Austria.
Tel.: +43 512 507 3451; Fax: +43 512 507 2861;
E-mail: gottfried.baier@uibk.ac.at
[4]These authors contributed equally to this work

Received: 19 May 2005; accepted: 7 October 2005; published online: 27 October 2005

PKCθ and Akt/PKBα in T cells. Thus, our current findings expand our understanding of the role of antigen receptor-induced autophosphorylation, which contributes to the specific function of PKCθ in T cells.

Results

Identification of the predominant activation-induced autophosphorylation site of PKCθ as Thr-219

Pilot studies demonstrated that recombinant PKCθ undergoes autophosphorylation, as assessed by an *in vitro* kinase assay performed in the absence of exogenous substrate. As the first step in assessing the major site(s) of phosphorylation of PKCθ, samples of *in vitro* autophosphorylated PKCθ were subjected to trypsin digest, HPLC fractionation and radio-sequencing of the fractions (Figure 1A). Results demonstrated that the major site of phosphorylation was likely two residues N-terminal to an Arg or Lys, since the signal was exclusively at position 2 in the most highly phosphorylated fraction of tryptic peptides (fraction 14) and substantially at position 2 in the other active fraction (fraction 5). Although there are 12 such Ser/Thr residues in PKCθ, our mutational analysis emphasized those Ser/Thr whose surrounding sequence was predicted to be least disfavorable to phosphorylation by PKC (Fujii *et al*, 2004), such as Thr-219 and Thr-210. Consistently, mutation of Thr-219 of PKCθ resulted in a complete defect of autophosphorylation (Figure 1B).

Tryptic peptide maps revealed that phorbol 12,13-dibutyrate (PDBu) stimulation of the recombinant isolated PKCθ *in vitro* caused an increase in ^{32}P content of several peptides (Figure 2A). Several dominant peptides were missing from corresponding maps of the T219A mutant (Figure 2B), confirming that Thr-219 was a phosphorylation site. Interestingly, and despite the fact that Thr-219 was the major autophosphorylation site of PKCθ, a short peptide thereof (as well as the other trans- and autophosphorylation sites, Thr-538, Ser-676 and Ser-695 in PKCθ) was not a suitable peptide substrate in *in vitro* PKCθ kinase assays (Figure 2C and D). The PKCθ-derived pseudosubstrate peptide, used as positive control, was efficiently phosphorylated.

Confirmation of this novel phosphosite identification was derived from the generation of phospho-specific (p)Thr-219 antisera and site-directed mutagenesis: The specificity of the antiserum for (p)Thr-219 was confirmed by *in vitro* studies with mutant proteins overexpressed by baculovirus (Figure 3B). After addition of PDBu to the isolated proteins *in vitro*, the phosphopeptide antibody to Thr-219 strongly recognized PKCθ. Similarly, phosphopeptide antibody to Thr-538 and Ser-695 reacted in this epitope-specific manner. The antibody was then used to assess PKCθ phosphorylation in intact cells. The antiserum reacted strongly with endogenous PKCθ in PDBu-stimulated primary mouse CD3$^+$ cells, but not in unstimulated cells (Figure 3A). In intact Jurkat T cells, this Thr-219 phosphorylation site within the regulatory domain of PKCθ was also inducibly phosphorylated (Figure 3C).

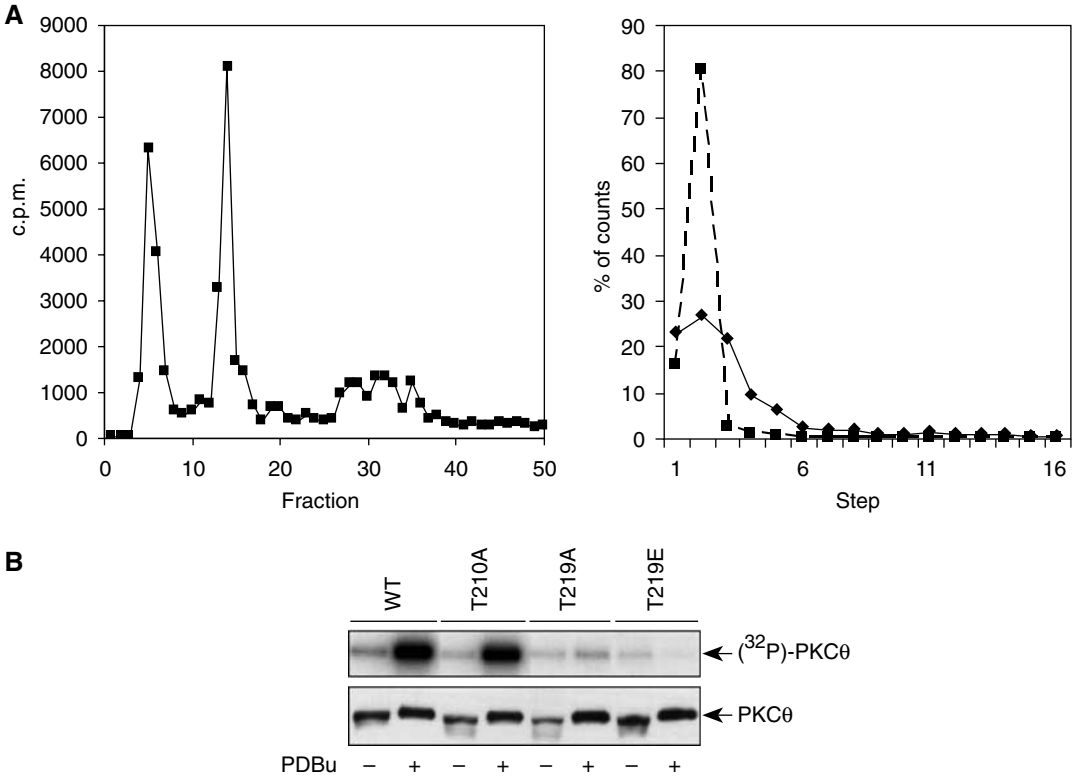

Figure 1 Dominant autophosphorylation of PKCθ. (**A**) PKCθ was autophosphorylated *in vitro*, digested with trypsin, fractions separated by HPLC and Cerenkov emissions measured (left panel). The major peaks of the digest (fractions 5, solid line and fraction 14, dashed line) were subjected to sequential release of residues by Edman degradation and released fractions assessed for β emissions measured in the presence of scintillant (right panel). (**B**) Transfected PKCθ wild-type or mutant cDNA constructs were immunoprecipitated with anti-tag antibodies and *in vitro* kinase assay performed in the presence of γ^{32}P-ATP with or without 100 nM PDBu stimulation, as indicated. Product was analyzed by SDS–PAGE, followed by autoradiography. Note that PKCθT219A and T219E mutants were mostly unable to autophosphorylate. Equal concentration of kinase in each lane was confirmed by immunoblotting.

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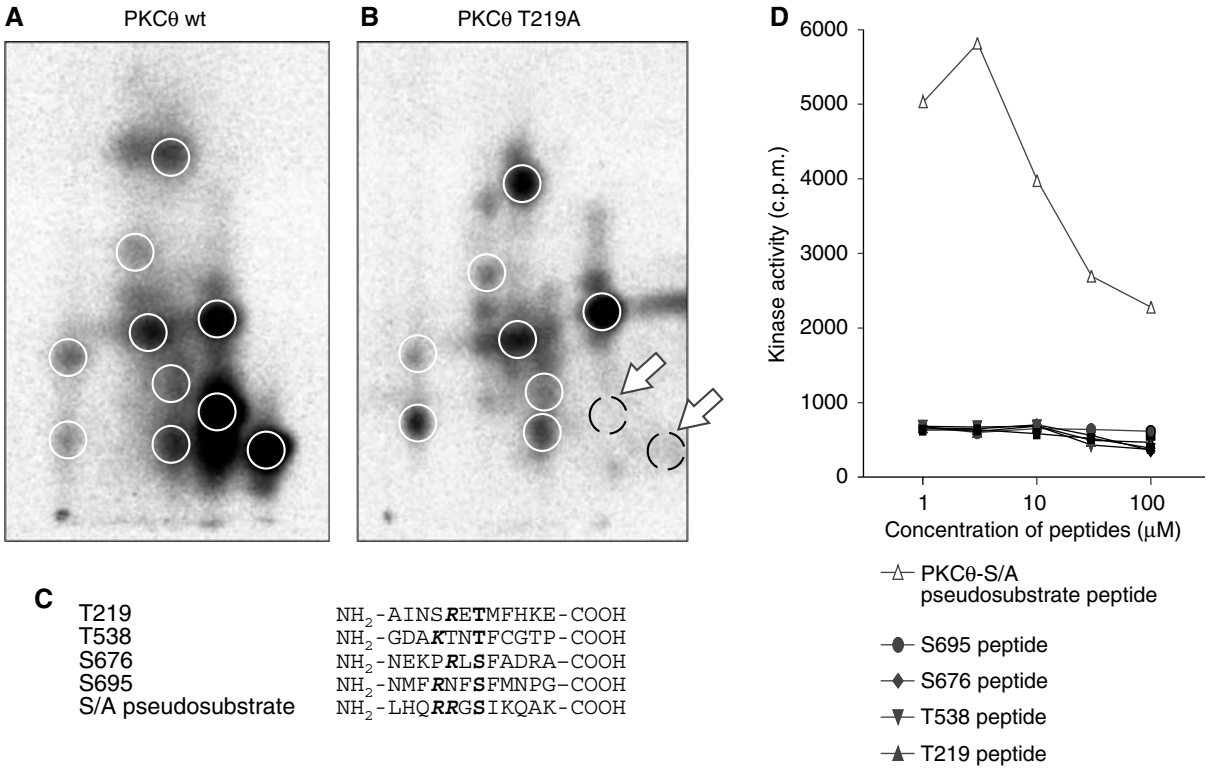

Figure 2 Thr-219 is a major contributor to autophosphorylation. Tryptic phosphopeptide maps of baculo-expressed and purified PKCθ wild-type (**A**) or PKCθ T219A mutant proteins (**B**). The circles depict the major peptides obtained from PKCθ. Note that two major phosphopeptides were missing in the mutant, as indicated by the arrows. (**C**) Amino-acid sequences of the peptide containing Thr-219, Thr-538, Ser-676 and Ser-695 of human PKCθ; phosphorylation site and NH_2-terminal basic residues are shown in bold. (**D**) Phosphorylation of these substrate peptides, employing recombinant baculo-expressed PKCθ, and scintillation proximity kinase assays are shown.

We have previously reported an autophosphorylation reaction of PKCθ, which was recognized by a broadly reactive phospho-Thr-specific antibody upon activation with PDBu or anti-CD3 crosslinking (Bauer *et al*, 2001); however, the particular residue recognized by the phospho-Thr antibody was not defined. The present study now defines the Thr-219 site as the major site in PKCθ. This previously described phospho-Thr-specific antibody bound the T219A mutant much less than wild-type PKCθ in Jurkat cells after PDBu stimulation (Figure 3D). Consistently, in immunoprecipitation analysis of transiently transfected Jurkat cell lysates, the new (p)Thr-219 antiserum reacted strongly with wild-type PKCθ, but not nearly as strong with the corresponding T219A mutant of PKCθ (Figure 3E). This residual (p)T219 staining of the T219A mutant thereby indicated crossreactivity of our polyclonal antiserum against another yet undefined, but PDBu-inducible, (p)Thr site within PKCθ.

Inhibitor studies provided corroboration for the hypothesis that Thr-219 was an autophosphorylation site. In primary $CD3^+$ T cells, PDBu-inducible phosphorylation on Thr-219 was prevented by preincubating cells with the pan-PKC inhibitor Gö6850, but was not prevented by the cPKC inhibitor Gö6976 (Figure 4A, upper panel). As a negative specificity control for our phosphopeptide Thr-219-specific antibody, PKCθ-deficient $CD3^+$ T-cell lysates derived from our established $PKC\theta^{-/-}$ mouse line (Pfeifhofer *et al*, 2003) did not reveal any band (Figure 4A, left two lanes). Studies in Jurkat cells confirmed these observations (Figure 4B). The fact that phosphorylation was not observed with the ATP-binding defective K409R mutant of PKCθ was consistent with our working hypothesis that Thr-219 was an autophosphorylation site. LY-294002, cyclosporine A and rapamycin (relatively specific inhibitors of PI-3 kinase, calcineurin (CaN) and the target of rapamycin kinase (mTOR), respectively) did not inhibit PDBu-induced Thr-219 autophosphorylation (data not shown). Taken together, these results strongly argue that autophosphorylation occurs at this site in PKCθ.

Next we tested whether antigen receptor activation induced Thr-219 phosphorylation of endogenous PKCθ in primary mouse and human T cells. Indeed, CD3/CD28 crosslinking resulted in significant phosphorylation of (p)Thr-219 in mouse $CD3^+$ cells (Figure 5A) and Jurkat cells (Figure 5B). Consistent with this finding, phosphorylation was also induced by vanadate, implying a critical upstream role of protein tyrosine phosphorylation. No activation was observed upon okadaic acid (a potent inhibitor of serine/threonine phosphatases) treatment of cells (Figure 5B). Furthermore, (p)Thr-219 induction was comparable with CD3 versus CD3 plus CD28 induction. The potential importance of Thr-219 in PKC signaling was further emphasized by the observation that Thr-219 was phosphorylated in primary human $CD4^+$ cells in response to PDBu, whereas two other known sites of PKCθ phosphorylation, Thr-538 and Ser-695, were both found to be constitutively phosphorylated under the experimental conditions used (Figure 5C). Thus, combined evidence from phosphopeptide mapping and from a phosphosite-specific antisera identified a novel and

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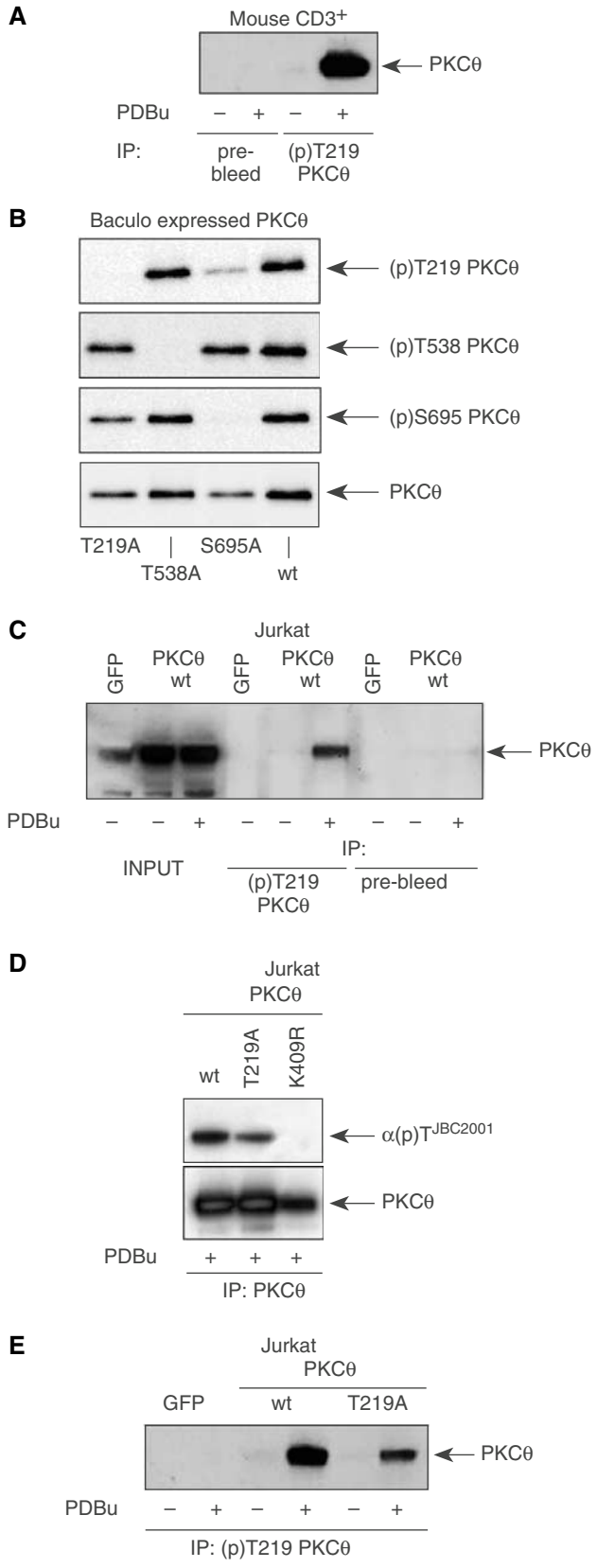
activation-inducible autophosphorylation site in PKCθ, (p)Thr-219. This site resides within the hinge region of the tandem C1 domains within the regulatory fragment of PKCθ.



PKCθT219A mutant is intact in its enzymatic activity in vitro

In order to determine whether phosphorylation of PKCθ at Thr-219 plays any role in the regulation or function of PKCθ, we first studied the T219A mutant in Jurkat cells. PKCθ variants were expressed, immunoprecipitated, normalized by their expression levels and analyzed for their kinase activities. As shown in Figure 6A, wild-type PKCθ and the PKCθT219A (as well as the acidic exchange mutant PKCθT219E) readily phosphorylated the peptide substrate

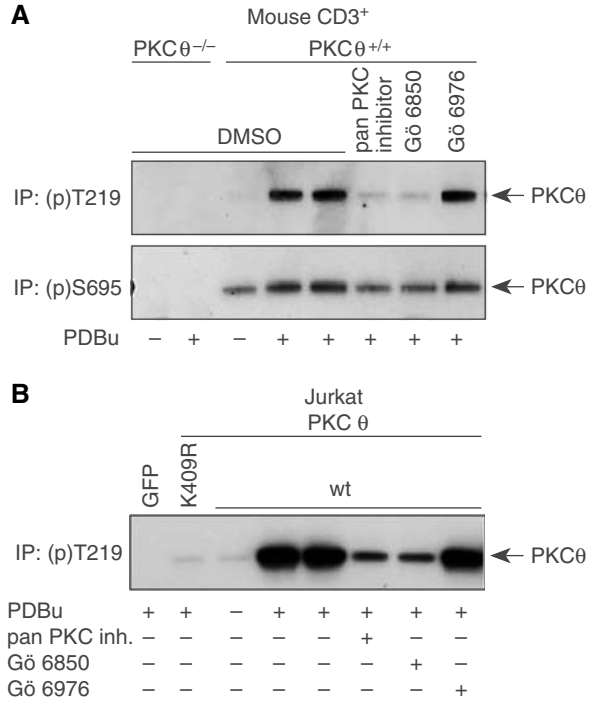


Figure 4 Thr-219 phosphorylation depends on PKCθ catalytic activity. PKCθ was immunoprecipitated from resting (−) or 20 min and 100 nM PDBu-stimulated (+) mouse CD3$^+$ cells, purified from lymph nodes and spleen (**A**), and Jurkat cells (**B**), transfected with GFP control, PKCθ wild type or PKCθK406R as indicated. The Thr-219 phosphostatus was determined as in Figure 3, except that the cells were pretreated for 1 h with distinct PKC inhibitors (at 500 nM concentrations) or DMSO buffer control, as indicated. In contrast, the classical hydrophobic motif autophosphorylation site of PKCθ, (p)S695, was constitutively phosphorylated and induced much less upon stimulation (A, lower panel). One representative result out of three independent experiments was shown.

Figure 3 Thr-219 phosphorylation in PKCθ is inducible. (**A**) PKCθ was immunoprecipitated from resting (−) or 20 min and 100 nM PDBu-stimulated (+) primary CD3$^+$ mouse cells and immunoblotted with PKCθ-specific mAb. (**B**) Baculo-expressed and isolated PKCθ mutant proteins were tested with phosphopeptide-specific antibodies, (p)T219, (p)T538 and (p)S695 on total wild-type and mutant PKCθ proteins to confirm their phosphoepitope specificity. (**C**) The stoichiometry of the Thr-219 site-specific phosphorylation of PKCθ was estimated with immunoprecipitation employing the (p)Thr-219-specific antibody versus total recombinant PKCθ protein (INPUT) from PKCθ wild-type cDNA transfected cell lysates. This procedure was described in Miinea and Lienhard (2003). (**D, E**) Jurkat T cells, transiently transfected with GFP inert protein control, PKCθ wild type or PKCθT219A or PKCθK406R, as indicated were immunoblotted with PKCθ-specific mAb. One representative result out of three independent experiments was shown.

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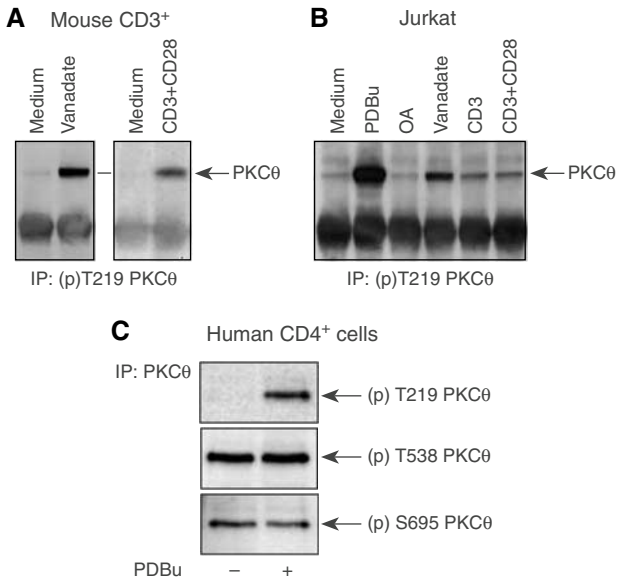

Figure 5 Phosphostatus analysis of PKCθ. Thr-219 phosphorylation of endogenous PKCθ in primary CD3$^+$ cells (**A**), in Jurkat cells, transfected with PKCθ wild-type cDNA (**B**) and in primary human CD4$^+$ cells (**C**), treated with medium alone, solid-phase CD3 and/or CD28 antibodies, 100 nM PDBu, 200 μM pervanadate or 1 μM okadaic acid for 20 min at 37°C. Similar results were obtained in at least three independent experiments.

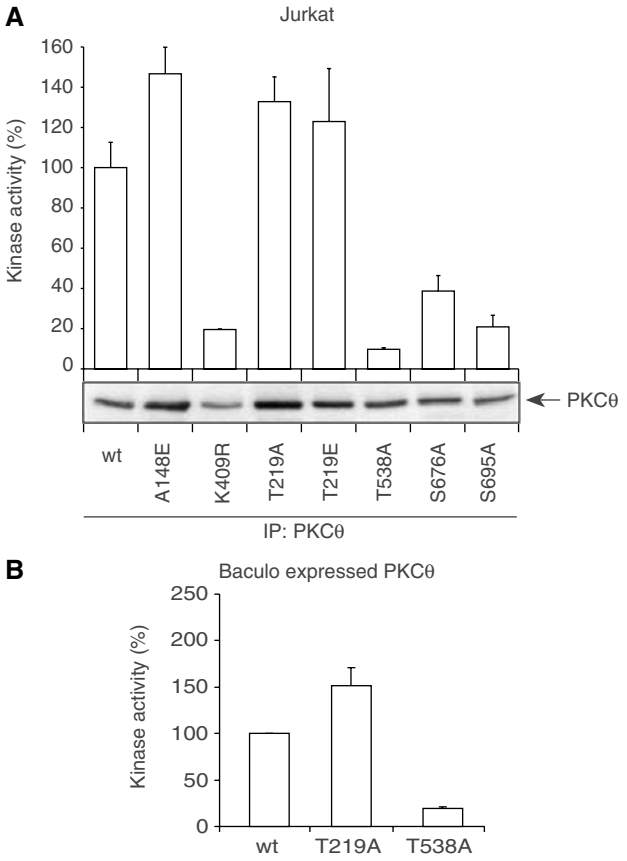

Figure 6 Thr-219 is not required for the catalytic activity of PKCθ. (**A**) *In vitro* kinase assay of PKCθ immunoprecipitates from Jurkat cells transfected with PKCθ cDNA expression plasmids as indicated. The upper panel shows the ability of PKCθ to phosphorylate the pseudosubstrate peptide, and the lower panel represents the corresponding anti-PKCθ immunoblots. The kinase activity thereby has been normalized for each PKCθ mutant protein by the expression levels, and results expressed relative to that measured with the wild-type protein. (**B**) Similar experiment as in panel A, except that baculo-expressed and purified PKCθ protein was used as the source ($P<0.005$ in (A) and (B); *t*-test).

in vitro. In contrast, PKCθ exhibited reduced activity in mutant constructs lacking phosphorylation sites at Thr-538, Ser-676 and Ser-695. As a negative control, PKCθK409R, an ATP-binding defective and thus kinase-inactive mutant, was used. Reduction in enzymatic activity was especially notable in the activation loop mutant T538A, as defined previously elsewhere (Liu *et al*, 2002; Xu *et al*, 2004).

Baculo-expressed recombinant PKCθT219A mutant also had normal kinase activity, when compared to wild-type PKCθ (Figure 6B). Since the enzymatic activity was intact and, even slightly enhanced, this excludes the possibility that mutation of Thr-219 had an overall negative effect on the folding or kinase function of PKCθ. Therefore, and in contrast to mechanisms proposed for the established autophosphorylation sites within the catalytic domain of PKCθ, this novel (p)Thr-219 site does not inhibit catalytic activity *in vitro*.

Phosphorylation at Thr-219 is required for transactivation of the IL-2 promoter

To test whether phosphorylation at Thr-219 is important for the function of PKCθ in TCR-mediated signal transduction, we expressed wild-type and T219A mutant PKCθ constructs in Jurkat T cells together with a luciferase reporter gene driven by the entire IL-2 promoter. Here we employed the constitutively active (CA) mutant CA-PKCθA148E (Baier-Bitterlich *et al*, 1996), which, in combination with ionomycin, was able to strongly induce transactivation of downstream transcription factors in transfected cells. The CA-PKCθT219A mutant was expressed at levels similar to the wild-type protein, as analyzed by immunoblotting (Figure 7A, inset). While cells transfected with the reporter gene alone did not markedly respond to ionomycin stimulation, cells cotransfected with CA-PKCθ exhibited an enhanced activation of the IL-2 promoter (Figure 7A). In contrast, the CA-PKCθT219A, K409R, T538A and S676A mutants failed to demonstrate enhanced IL-2 promoter activation as compared to the endogenous signaling seen in the GFP inert protein transfection control. Only the CA-PKCθS695A mutant demonstrated slightly enhanced IL-2 transactivation.

These results were confirmed by a modified activation protocol in which transfection with constitutively activated mutant of CaN was used instead of ionomycin stimulation (Figure 7B). In accordance with earlier data, the T219A mutant of CA-PKCθ (as well as T538A, S676A and S695A) completely failed to mediate NF-AT transactivation in Jurkat T cells under these conditions. Finally, the T219A mutant of CA-PKCθ (as well as S676A and S695A) lost its capacity to mediate NF-κB transactivation upon CD3/CD28 crosslinking in Jurkat T-cells (Figure 7C). Thus, the T219A mutant was as defective as kinase-dead mutants (K409R and T538A) in activation of transcription via the intact IL-2 promoter or from NF-AT and NF-κB binding elements.

Another useful readout of PKCθ function was its phosphorylation of endogenous substrates in transiently transfected Jurkat cells, as measured by immunoblot with an anti-(p)Ser substrate antibody (Figure 7D); expression of the CA-PKCθ

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kinase reproducibly induced the phosphorylation of a large array of proteins in the cytosolic fraction. This may indicate that in addition to few substrates directly phosphorylated by PKCθ there are many additional phosphorylation events, induced via PKCθ-mediated downstream kinase cascades. The CA-PKCθT538A activation loop mutant demonstrated dramatically reduced cellular substrate phosphorylation. No PKCθ-dependent substrate phosphorylation was detectable after transfection with ATP-binding defective PKCθK409R mutant (when compared to the GFP transfection control). Strikingly, the CA-PKCθT219A mutant also demonstrated a significantly reduced ability to phosphorylate these endogenous substrates. Taken together, these results indicate that expression of a nonphosphorylatable Thr-219 motif mutant of PKCθ in T cells markedly diminishes its ability to activate downstream signaling in response to cellular activation in intact cells, and therefore indicates that phosphorylation of Thr-219 may be essential for the proper function of PKCθ in antigen receptor-mediated signal transduction.

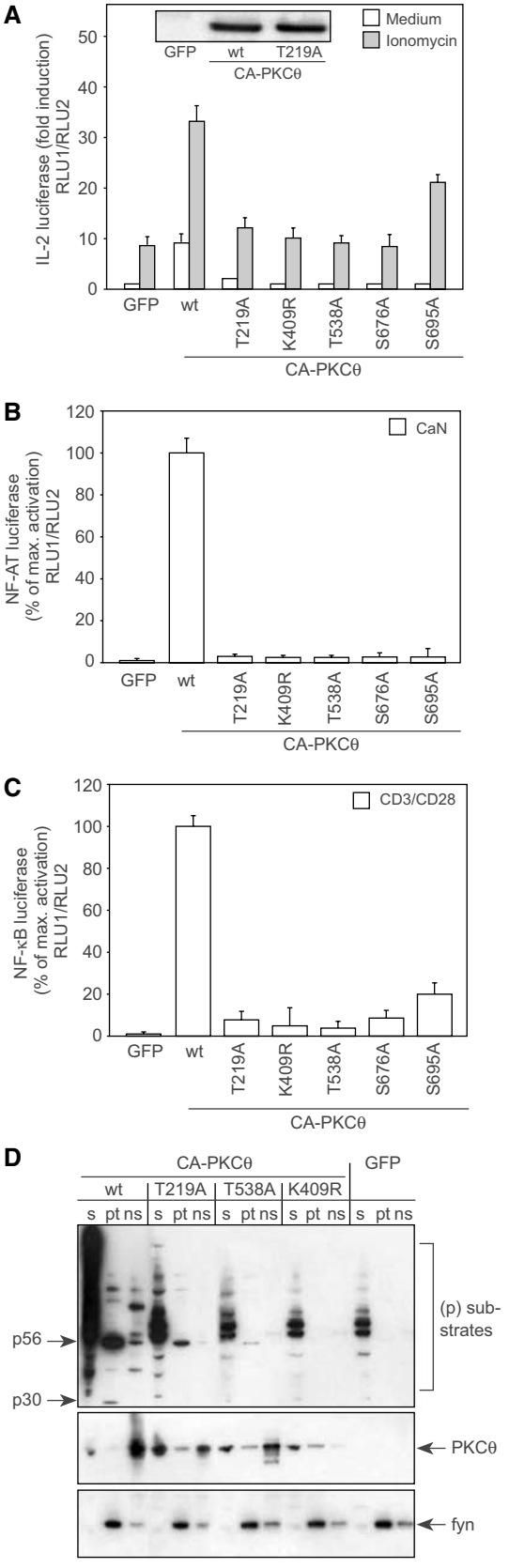

Figure 7 Thr-219 is required for the activity of PKCθ in Jurkat T cells. (**A–C**) Jurkat cells transfected with GFP inert protein control or CA mutants of PKCθ and CaN as indicated. Expression of the transgenes has been confirmed by immunoblotting of equal amounts of total cell lysates (inset). Luciferase activity in lysates of cells cotransfected with the indicated PKCθ expression constructs, luciferase reporter plasmid, and either left unstimulated or stimulated with 500 nM ionomycin or solid-phase CD3/CD28 antibodies for 16 h at 37°C. The measured firefly luciferase activity was normalized for transfection efficiency using the renilla luciferase activity, and results were expressed relative to that induced by the unstimulated GFP control. Values represent the average of at least three experiments carried out in duplicates ($P<0.005$ in (A–C); *t*-test). (**D**) Transiently transfected Jurkat T cells were subjected to biochemical fractionation and subsequent immunodetection with a broadly reactive anti-phosphoserine PKC substrate antibody (able to detect distinct phospho-Ser containing endogenous proteins): biochemical fractions corresponding to cytosol (s), membrane (pt) and cytoskeletal fractions (ns) were prepared by standard fractionation, resolved by SDS–PAGE, and the phospho-Ser PKC substrate staining of cellular proteins. Additionally, recombinant expression and relative subcellular distribution of the PKCθ mutants (in comparison to the GFP vector control) was determined. As positive evaluation for cell fractionation, immunostaining of $p59^{fyn}$ predominantly recognized the pt fraction. The results are representative of three experiments.

PKCθT219A is not enriched at the plasma membrane

In order to investigate the molecular mechanism involved, we determined whether the T219A mutant was able to translocate into the plasma membrane upon cellular activation, employing immunofluorescence staining of transiently transfected Jurkat cells. Once stimulated with superantigen stapphylococcal enterotoxin A (SEA) (presented by Raji B cells), translocation of PKCθ protein to the T- and B-cell contact zone was reproducibly observed in the PKCθ wild-type, but not in the PKCθT219A mutant, transfected cells (Figure 8A).

Since the endogenous PKCθ protein interferes with studies of transfected PKCθ in Jurkat cells, we next confirmed this observation biochemically. Anti-CD3/CD28-stimulated CA-PKCθ-transfected Jurkat cells were therefore fractionated by sucrose gradient centrifugation to separate the buoyant, detergent-insoluble, glycolipid-enriched lipid rafts from the detergent-soluble fractions of the cells. As reported (Bauer *et al*, 2003), the CA mutant of PKCθ was found to be strongly enriched in the lipid rafts when expressed and activated in

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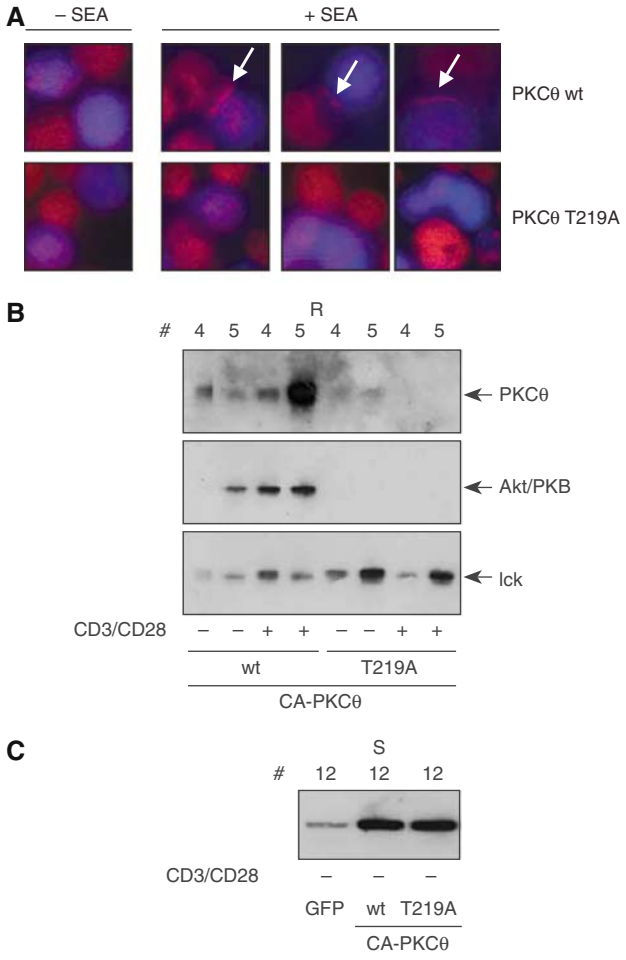

Figure 8 Thr-219 is required for submembranous location of PKCθ in Jurkat T cells. (**A**) Jurkat cells were transfected with PKCθ wild-type or T219A mutant, as indicated, and 21 h later incubated with Raji B cells (stained in blue) in the absence or presence of SEA for 15 min. Recombinant PKCθ was then stained in red by immunofluorescence. Representative images out of three independent experiments are shown. The arrows mark PKCθ enrichment in the T- and B-cell contact zone. (**B, C**) Jurkat T cells transfected with CA-PKCθ wild-type or CA-PKCθT219A cDNA, respectively, as indicated. Lipid raft fractions (R) and detergent-soluble fraction (S) were immunostained for PKCθ and Akt/PKB in order to differentially contrast protein lipid raft distributions of the same cells. As a marker for the raft fraction, $p56^{lck}$ was used for immunoblots of the same samples. A representative experiment of three independent experiments was shown.

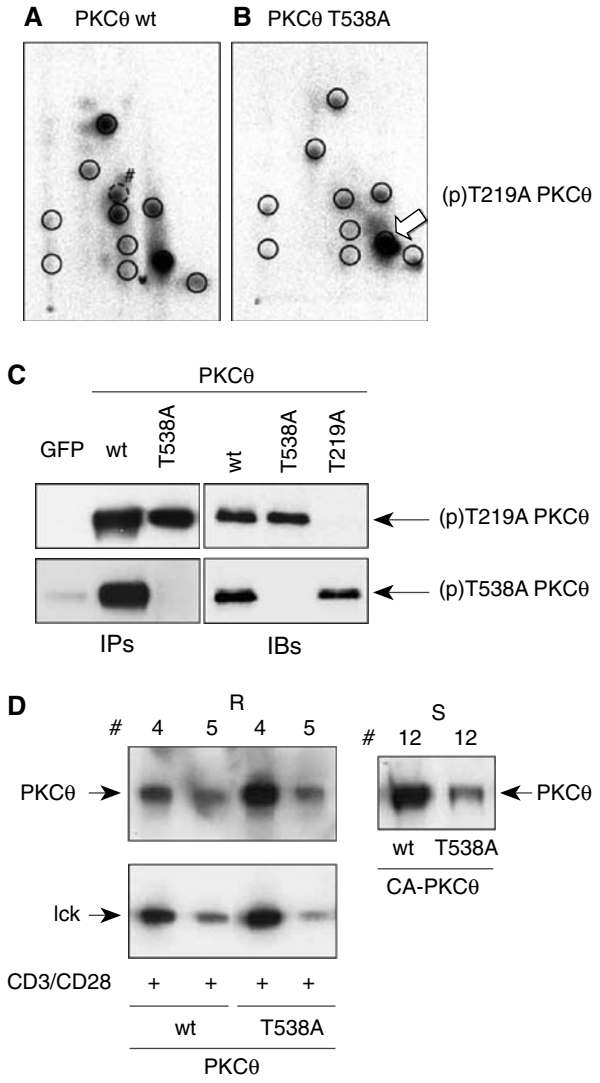

Figure 9 'Kinase-low' Thr-538 activation loop mutant is phosphorylated at Thr-219 and demonstrates intact submembranous location of PKCθ in Jurkat T cells. Tryptic phosphopeptide maps of baculo-expressed and purified PKCθ wild-type (**A**) or PKCθT538A mutant proteins (**B**). The circles depict the peptides obtained from PKCθ. The major (p)Thr-219 representing phosphopeptide was indicated by the arrow. (**C**) Recombinant wild-type and mutant PKCθ was immunoprecipitated (IPs—left panel) from PDBu-stimulated Jukat cells or purified from baculovirus-infected Sf21 cells (IBs—right panel) and immunoblotted with the phosphopeptide-specific antibodies, (p)T219 and (p)T538, as indicated. (**D**) Jurkat T cells transfected with CA-PKCθ wild-type or CA-PKCθT538A cDNA, respectively, as indicated. Lipids raft fractions (R) and detergent-soluble fraction (S) were immunostained for PKCθ and $p56^{lck}$.

Jurkat T cells (Figure 8B). Immunoblot detection of the tightly raft-associated $p56^{lck}$ provided a positive control for the raft fractionation. However, when the potential raft location of CA-PKCθT219A mutant was analyzed following CD3/CD28 ligation, striking differences were revealed: immunoblots of these fractions demonstrated that, again, this T219A mutant was not induced to redistribute into rafts following CD3/CD28 stimulation (Figure 8B). Differences in cellular location observed were not due to altered expression levels, since the recombinant expression levels of both forms of PKCθ in cytosolic fractions of resting cells were nearly identical (Figure 8C). These results, which were observed via independent methodology, thus indicate that the T219A mutation impaired the correct targeting of PKCθ upon TCR ligation.

Interestingly, the PKCθT538A mutant, which was found to be enzymatically inactivated (Figure 6A and B above), was still able to autophosphorylate at Thr-219 (Figure 9A–C). One potential explanation is that the T538A mutant was not completely kinase-dead but instead had severely reduced catalytic activity. Consistently with the intact Thr-219 autophosphorylation, the T538A mutation did not affect targeting of CA-PKCθ to its submembranous location (Figure 9D).

One possible explanation for the defect in its submembranous translocation could be that the lipid binding by the regulatory domain was altered by the Thr-219 mutation.

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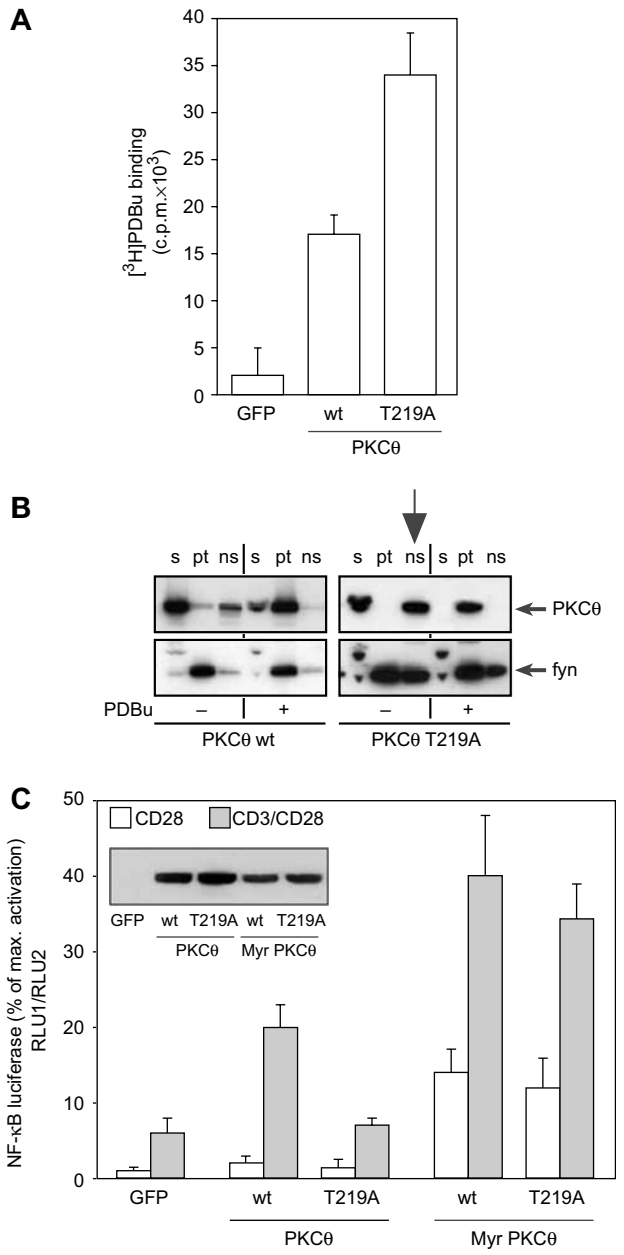

Figure 10 Thr-219 is not required for PDBu binding in Jurkat T cells. Jurkat T cells were transfected with PKCθ wild-type or PKCθT219A cDNA expression vectors, as indicated. After 21 h, cells were (**A**) determined for specific ^{3}H-PDBu binding as described (Chida and Kuroki, 1983) and (**B**) stimulated with 100 nM PDBu for 20 min or left unstimulated, as indicated. Subcellular distribution of PKCθ (upper panel) and p59^{fyn} (lower panel) was then determined by immunoblotting. The cell fractions were defined as the soluble (s) fraction, the particulate (pt) fraction and the Triton X-100 nonsoluble (ns) fraction. Note the constitutive enrichment of the T219A mutant in the ns fraction of nonstimulated cells (indicated by the arrow). (**C**) MyrPKCθT219A synergizes with CD3/CD28 in mediated NF-κB reporter activation. cDNA expression vectors encoding GFP control, wild-type and Myr-NH$_2$-terminal fusion mutants of PKCθ, respectively, together with the NF-κB reporter plasmid have been cotransfected in Jurkat T cells. After 21 h, cells were stimulated and assayed for reporter gene expression. Expression of the PKCθ transgenes has been confirmed by immunoblotting (inset).

However, PDBu binding was not only impaired but even slightly enhanced in the T219A mutant (Figure 10A). Analysis of subcellular fractionation revealed that the T219A mutant, similarly to wild-type PKCθ, was able to translocate into the plasma membrane upon PDBu stimulation, confirming intact PDBu binding in intact cells (Figure 10B). Interestingly, however, in nonstimulated cells, the T219A was enriched in the Triton X-100 nonsoluble (ns) fraction, which again indicated improper subcellular targeting of this PKCθ mutant protein.

To directly address the question that default targeting of PKCθT219A to a submembranous location may be responsible for the impaired recruitment and activation of the kinase upon TCR stimulation, we created constitutively plasma membrane-targeted forms of PKCθ and its T219A mutant by adding the first 16 amino acids of p56^{lck} to their NH$_2$-termini, as previously carried out for the wild-type enzyme (Bauer *et al*, 2001). This short sequence contains the necessary motifs to drive myristoylation and palmitylation of the proteins. Indeed, these membrane-targeted mutants, wild-type as well as T219A variant of PKCθ (MyrPKCθ), were found to be constitutively associated with submembranous structures (not shown). As observed in Figure 10C, the membrane-targeted PKCθT219A (MyrPKCθT219A) now responded to CD28 and CD3/CD28 stimulation, similarly to MyrPKCθ wild type, by increased transactivation of the NF-κB reporter. Thus, MyrPKCθT219A had largely regained the ability to participate in TCR signaling. The impaired activation and function of PKCθ caused by replacement of Thr-219 by an Ala can be substantially overcome by forced plasma membrane targeting of the mutant PKCθT219A.

As Akt/PKB and PKCθ interact and cooperate during CD3-mediated activation, we also analyzed the location of Akt/PKB. Using biochemical fractionation assays, we found that the impaired recruitment of CA-PKCθT219A mutant also affected endogenous Akt/PKB recruitment into the raft lipid microdomain (Figure 8B above). Following up on this observation, we investigated whether phosphorylation of Thr-219 was critical for the established crosstalk between PKCθ and Akt/PKBα in T cells. While overexpression of both CA-PKCθ and Akt/PKBα wild type synergistically increased the activation of NF-κB enhancer (Figure 11A and; Bauer *et al*, 2001), coexpression of CA-PKCθT219A mutant with Akt/PKBα yielded a level of NF-κB activation similar to that of control vector transfected cells. Similarly, a ΔPH mutant of Akt/PKBα was incapable of cooperating with CA-PKCθ (Figure 11A). Moreover, T219A mutation abrogated the physical association of CA-PKCθ with endogenous Akt/PKB (Figure 11B) assessed by coimmunoprecipitation. Physical association of CA-PKCθ with endogenous Akt/PKB in Jurkat cells, however, was not dependent on cellular activation (Figure 11B; Bauer *et al*, 2001), but strictly dependent on the presence of the PH domain in Akt/PKBα, since the PH-deletion mutant of Akt/PKBα was incapable of interacting with CA-PKCθ (Figure 11C).

This indicates that the Thr-219 phosphorylation of PKCθ may be an intrinsic structural requirement, indispensable for the physical and functional interaction with Akt/PKB (for a review, see Bauer and Baier, 2002). Coexpression of the membrane-targeted PKCθT219A (MyrPKCθT219A) mutant with Akt/PKBα again rescued the ability to support signaling via Akt/PKBα to the NF-κB enhancer, similarly to MyrPKCθ wild type (not shown). This indicates that proper subcellular targeting of PKCθ is a key feature to physically and functionally interact with Akt/PKB. Based on these experiments, we

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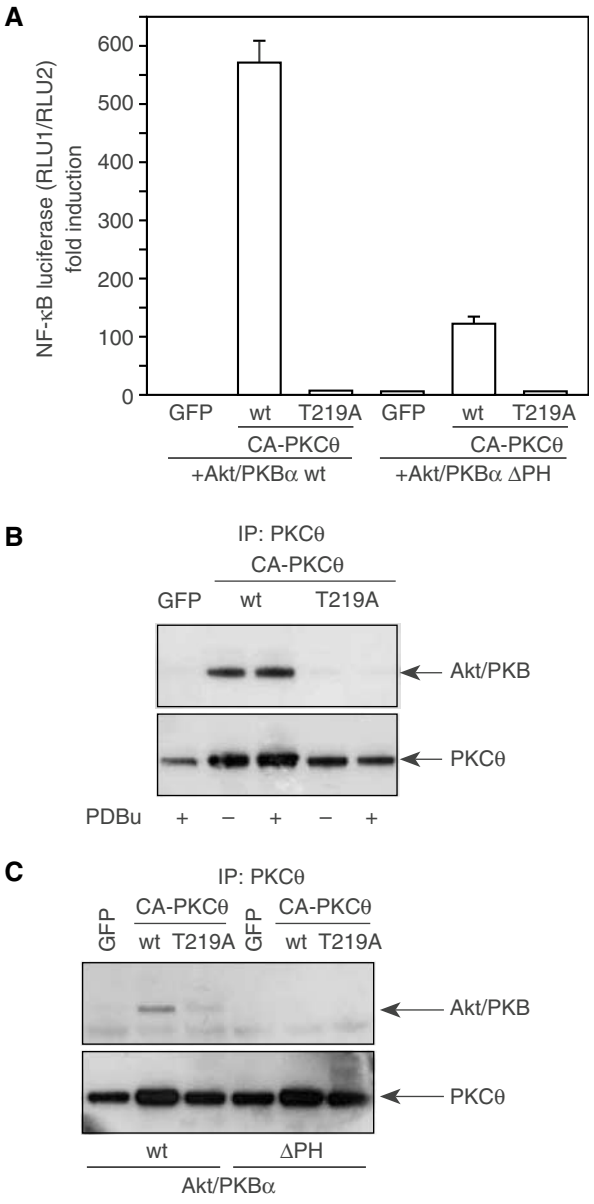

Figure 11 Thr-219 is required for PKCθ crosstalk with Akt/PKBα in T cells. (**A**) Jurkat T cells were transfected with CA-PKCθ and Akt/PKBα constructs, as indicated, stimulated for 16 h with 500 nM ionomycin and analyzed as in Figure 7. Statistical analysis of three independent experiments was evaluated as the means of fold induction ± s.e.; $P<0.005$. Expression of the transgenes has been confirmed by immunoblotting (not shown). (**B**) Coimmunoprecipitation analysis of recombinant PKCθ and endogenous Akt/PKB. PKCθ immunoprecipitations of Jurkat T-cell lysates, transfected 21 h earlier with GFP control, CA-PKCθ or CA-PKCθT219A expression vectors (as indicated). Jurkat T cells were stimulated for 20 min at 37°C with 100 nM PDBu or left unstimulated. Afterwards, cell extracts were immunoprecipitated (IP) with an anti-PKCθ antibody. (**C**) Coimmunoprecipitation analysis of recombinant PKCθ and Akt/PKBα in nonstimulated Jurkat T-cell lysates, transfected 21 h earlier with GFP control, CA-PKCθ or CA-PKCθT219A and Akt/PKBα wild-type and PH domain deletion mutant (ΔPH) expression vectors (as indicated). Immunoblots were stained for PKCθ (lower panel) and Akt/PKB (upper panel), respectively. Comparable results were obtained in two independent experiments.

conclude that autophosphorylation of PKCθ at Thr-219 plays a role in proper protein:protein interaction and intracellular targeting of PKCθ in T cells.

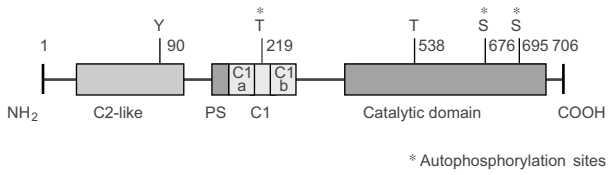

Figure 12 Cartoon of PKCθ domain structure and phosphorylation sites. The domain structure of PKCθ was schematically shown and consists of a conserved serine/threonine kinase (COOH-terminus) and a regulatory (NH2-terminus) domain. Thr-219 resides within the cystein-rich (C1) sequences of PKCθ, responsible for second-messenger lipid interaction. The established phosphorylation sites of serine (S), threonine (T), and tyrosine (Y) residues are indicated.

Discussion

The PKCθ serine/threonine kinase has been repeatedly implicated in lymphocyte activation, but there has been little precise understanding of the PKCθ isotype-selective mechanisms. Mechanism-based understanding of PKCθ depends on the characterization of its defined activation steps. Phosphorylation and second-messenger binding, two co-ordinated steps in the PKC activation process, are known to regulate PKC kinases. PKCs share three conserved phosphorylation motifs within their catalytic domain, the activation loop segment, the turn motif and the hydrophobic motif. Phosphorylation at each of these sites regulates the maturation, catalytic competence and thus cellular signaling function, including downregulation of PKC (Newton, 2003; Parker and Murray-Rust, 2004).

Using phosphopeptide mapping and mutagenesis-based hypothesis testing, we have identified a novel site of regulatory phosphorylation on PKCθ, Thr-219, which is located in the hinge region between the C1A and C1B domains, upstream of the catalytic domain of PKCθ (see the cartoon in Figure 12). Analysis of evolutionary sequence conservation revealed that, within the PKC family members, this sequence surrounding Thr-219 represents a motif, N S R D/E T M/I F, which was found only in the nPKC members of PKCδ and θ. However, PKCδ and θ have distinctly different functions in T-cell signaling (Gruber *et al*, 2005).

The present analysis showed that Thr-219 was the dominant site of *in vitro* autophosphorylation. Tryptic peptide maps of recombinant PKCθ *in vitro* confirmed that Thr-219-containing peptides from PKCθ contain phosphate (Figures 1 and 2). Phosphorylation of Thr-219 is likely to give rise to multiple spots because of the following reasons: (1) the surrounding sequence contains a Met that could also appear in an oxidized state; (2) partial cleavage may occur or (3) there may be additional phosphorylations that depend on prior phosphorylation at Thr-219. It should also be noted that a few additional sites of *in vitro* autophosphorylation remained to be defined in this phosphopeptide analysis of PKCθ, most likely including the established Ser-676 and Ser-695 sites. Nevertheless, Thr-219 was the major *in vitro* autophosphorylation site of PKCθ. The short peptide thereof (and the other autophosphorylation sites, Ser-676 and Ser-695 in PKCθ) was not an efficient peptide substrate in *in vitro* PKCθ kinase assays (Figure 2D). The pseudosubstrate contains an Arg- and Lys-rich motif, which resembles the PKC consensus sequence. In contrast, the motif of the autophosphorylation sites contained only one Arg or Lys in position

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−2 or −3. Additionally, the Thr-219 also had a disfavored acidic residue in position −1 (Fujii *et al*, 2004). Thus, in cases when the frequency of encounter between kinase and substrate was extremely high, as demonstrated with autophosphorylation sites within the full-length protein kinase, high proximity was most likely dominating over markedly suboptimal substrate consensus sequences (Zhu *et al*, 2005).

In intact cells, the ATP-binding defective PKCθK409R mutant or, alternatively, the pharmacologically inhibited PKCθ was not phosphorylated at Thr-219 upon cellular stimulation (Figure 4). Thr-219 phosphorylation was inducible both by mitogenic phorbol ester treatment and by CD3 antigen receptor crosslinking (Figure 5). Importantly, since experiments were conducted also with endogenous PKCθ in primary T cells (Figures 4 and 5), phosphorylation of Thr-219 appeared to be a physiologically relevant consequence of antigen receptor engagement. Since phosphorylation of Thr-219 required PKC activation, it was reasonable to hypothesize that this activation also resulted in phosphorylation of other physiologically relevant substrates in its vicinity. Consistently, the T219A mutation significantly reduced its ability to phosphorylate endogenous cellular substrates in intact Jurkat T cells (Figure 7D). Importantly, however, the *in vitro* catalytic activity was intact in T219A mutant (Figure 6). Thus, the PKCθT219A mutant was catalytically intact *in vitro*, but impaired in its cellular function. Indeed, this phosphorylation site proved to be important for the normal function of PKCθ, namely for the activation of NF-AT, NF-κB and, subsequently, IL-2 gene activation in the Jurkat cell line (Figure 7A–C). Our data suggested that phosphorylation at Thr-219 may be involved in properly localizing PKCθ within the cell following antigen receptor ligation. Alternatively, changes in the Thr-219 phosphorylation state may simply have altered PKCθ protein half-life during its submembranous location. In any case, the T219A PKCθ mutant was not found to be properly located in the T- and B-cell contact zone, as well as T-cell submembranous fractions (Figure 8), indicating that the change in the phosphorylation state of this residue played a pivotal role in the overall control of localization of this kinase. Interestingly, Thr-219 was found to be autophosphorylated even in the catalytic activity-low mutant T538A (Figures 6 and 9A–C). Consistently, the T538A mutant did not show any defect in subcellular targeting (Figure 9D), indicating that submembranous location of PKCθ seemed to be modulated by activation-dependent Thr-219 autophosphorylation, but not solely by PKCθ's catalytic competence. The exact mechanisms as well as the protein-binding partner of PKCθ, however, remained undefined.

Thus, Thr-219, located in the linker between the tandem C1 domains of the regulatory fragment in PKCθ, was an *in vitro* and *in vivo* autophosphorylation site in T cells. Autophosphorylation of PKC within the C1 domains had not been identified previously. Another activation-dependent autophosphorylation of related PKC family members on serine and threonine residues outside the catalytic domain had been reported in the C2 domain of PKCε (Pepio and Sossin, 1998) and PKCα (Ng *et al*, 1999). In the C1 domain of PKCε, a site close to the Thr-219 in PKCθ was established to regulate subcellular location and binding to actin (Prekeris *et al*, 1996, 1998); however, in PKCε, these cellular functions were regulated by an allosteric switch and not by phosphorylation. Thus, these two regions in PKCθ and PKCε are in identical locations within the proteins and are both used for targeting. Therefore, it is plausible that the hinge region between C1A and C1B is an important region for all PKC family members contributing to temporal, spatial and, ultimately, isotype-specific regulation.

Our interpretation of these *in vitro* data is that PKCθ activation is a complex, multi-step process, and it is likely that the tandem C1 domain has multiple, temporally distinct roles. The tandem C1 domain plays an essential role in second-messenger lipid binding early in the activation sequence of PKCθ. Phosphorylation of Thr-219 then follows as a second step and this may be a prerequisite for PKCθ to interact with its adaptor and scaffold molecules. As one potential mechanism, (p)Thr-219 site could serve to strengthen submembranous association and hinder dissociation of PKCθ from a multi-protein complex. Scaffold proteins such as WD40 containing 'RACKs' are candidate-targeting modulators, that is, phosphorylation at Thr-219 may promote binding to another cellular protein at the plasma membrane, which then serves as an anchor in this location. This possibility was supported by our finding that a construct with forced submembranous location mostly restored the ability of the PKCθT219A mutant to mediate CD3/CD28 signaling (Figure 10C). Consistent with this concept, our results identified an association between PKCθ and Akt/PKB and revealed (p)Thr-219 as an intrinsic structural determinant required for functional crosstalk between PKCθ and Akt/PKB in the TCR/CD28 signal transduction, which involves NF-κB activation (Figure 11). This was consistent with the hypothesis that corecruitment of both PKCθ and Akt/PKB to the plasma is a critical mechanism leading to activation of the NF-κB pathway (for a review, see Bauer and Baier, 2002). Indeed, the observed defect in binding of Akt/PKB and PKCθT219A in coimmunoprecipitation experiments (Figure 11B and C) and in submembranous recruitment of both PKCθT219A and Akt/PKB (Figure 8B) strongly argued in favor of this hypothesis.

We conclude that the primary role of Thr-219 phosphorylation is to target the kinase to the proper membrane microdomain, rather than to regulate its enzymatic activity. Phosphorylation at Thr-219 may promote binding to a multi-protein complex at the plasma membrane, which then serves as an anchor in this location. Consistently, the regulation of signal transduction leading to the activation of the IL-2 promoter depends on (p)Thr-219. Thr-219 phosphorylation thus is an important component of PKCθ's isotype-specific function during T-lymphocyte signal transduction.

Materials and methods

Reagents and plasmids

Gö6850, Gö6976, LY333531 and rapamycin were purchased from Alexis, Lausen, Switzerland. γ^{32}P-ATP was purchased from DuPont NEN, and PDBu and ionomycin from Sigma, both Vienna, Austria. The anti-Akt/PKB antibody was obtained from Cell Signaling Techn., USA. The antibodies used for PKCθ were obtained from Santa-Cruz, USA (#sc-1875) and Becton-Dickinson (#1520), respectively. The polyclonal (p)T219 PKCθ antibody was raised against (phospho)threonine-containing peptide sequences, NH_2- I N S R E (p)T^{219} M F H K E -COOH, coupled to KLH. Site-directed mutagenesis was performed using the QuikChange kit (Stratagene, La Jolla, CA), following the manufacturer's instructions. Recombinant baculovirus-expressed protein of PKCθ wild type and mutants was expressed in Sf21 cells and purified with Ni-NTA-agarose (Quiagen Inc., USA).

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Cells and transfections

Jurkat T cells were maintained in RPMI medium supplemented with 10% FCS (Life Technologies, Inc.). Transient transfection of cells was performed by electroporation in a BTX-T820 ElectroSquarePorator (ITC, Biotech, Heidelberg, Germany) apparatus using predetermined optimal conditions: 2×10^7 cells at 450 V/cm and five pulses of 99 ms. Optimix medium (Equibio, Kent, UK) was used for studies of promoter reporter gene expression. Reporter gene expression was measured in cotransfection assays using 10 μg pSRα-CD28, 15 μg of the expression vector and 15 μg of the relevant promoter firefly luciferase reporter (RLU1), as described (Bauer *et al*, 2001). For normalization, 0.3 μg of the renilla luciferase reporter vector pTK-Renilla-Luc (Promega, Madison, WI) (RLU2) was used. After 21 h, cells were stimulated with 100 nM PDBu and 500 nM ionomycin or solid-phase anti-CD3 and/or anti-CD28 for 16 h or left unstimulated, as indicated.

Cell fractionation

1×10^7 Jurkat T cells per assay point were transfected with 5–20 μg of the various cDNA expression plasmids, encoding wild-type or mutant PKCθ or empty vector controls, as indicated. Cell fractionation was performed by lysis in equivalent amounts of different buffers (without detergent—soluble, s fraction; containing 1% NP-40—particulate, pt fraction; containing 2% SDS—nonsoluble, ns fraction), as described (Bauer *et al*, 2001).

Coimmunoprecipitation analysis

1×10^7 T cells were stimulated with solid-phase antibodies against CD3 and/or CD28 for 15 min at 37°C or left unstimulated, and subsequently lysed in 1 ml of 5 mM Na_3VO_4, 5 mM Na-pyrophosphate, 5 mM NaF, 5 mM EDTA, 50 mM NaCl, 2% NP-40, 50 mM HEPES, pH 7.4, 50 μg/ml aprotinin and leupeptin. Lysates were precleared for 30 min at 4°C. Immunoprecipitation was performed at 4°C overnight using relevant antibodies. Thereafter, incubation with protein G Sepharose (Amersham-Pharmacia, Vienna) for 1 h at 4°C, extensive washing in lysis buffer, SDS–PAGE and immunostaining for the relevant protein were carried out.

Protein kinase scintillation proximity assay

Immunoprecipitates of recombinant overexpressed PKCθ constructs or, alternatively, purified baculovirus-expressed proteins were incubated in 50 μl kinase assay buffer (40 mM Tris, pH 7.5, 40 mM $MgCl_2$, 0.2 mM HEPES, pH 7.4, 0.2 mM DTT, 0.0002% Triton X-100, 0.3 mg/ml BSA) containing 1 μM ATP, 2 μCi [^{33}P-ATP], 3 μM of N-terminal biotinylated peptide (NH_2-RKRQRSMRRRVH-COOH), 1 μM PDBu and 160 μM phosphatidylserine. Similarly, biotinylated peptides of the Thr-219, Thr-538, Ser-676 and Ser-695 motifs were synthesized and used. PKCθ phosphorylation was measured by incorporation of $^{33}P_i$ from γ^{33}P-ATP, incubating 100 ng of the highly purified baculovirus-expressed recombinant proteins. After 40 min at room temperature, the reaction was stopped by adding 150 μl stop solution (10 mM ATP, 0.2 mg streptavidin-coated yttrium silicate beads (Amersham), 5 mM EGTA, pH 7.5, 0.1% Triton X-100). Radioactivity was counted with the Wallac Micro β1450 (Perkin-Elmer).

Phosphopeptide mapping

Recombinant PKCθ proteins were radiolabeled *in vitro* and subjected to SDS–PAGE. After autoradiography, the PKCθ band was excised from the corresponding area of the gel, digested to completion with trypsin and subjected to two-dimensional mapping. The procedure for radiosequencing was as described previously (Morrison *et al*, 1993).

Isolation of detergent-insoluble membrane (lipid raft) fractions

6×10^7 Jurkat TAg cells per assay point were transfected with 20 μg of the various cDNA expression plasmids, as indicated. After incubation for 21 h, the cells were stimulated with solid-phase IgG clones of CD3- and/or CD28-specific antibodies for 30 min at 37°C or left unstimulated. Subsequently, lipid rafts were collected as described (Bauer *et al*, 2003).

Immunofluorescence staining of Jurkat E6-1 T cells

Jurkat E6-1 cells were stimulated with SEA presented by Raji B cells. Raji cells were stained with cell tracker blue (Molecular Probes) and incubated with or without 2 μg of SEA per ml (Sigma) at 37°C for 90 min. 1×10^6 of Raji cells were then mixed with 1×10^6 transfected Jurkat E6-1 cells on polylysine-coated slides and incubated for 15 min at 37°C, fixed, permeabilized and stained with PKCθ goat antibody (Santa Cruz sc-1875) and subsequently with the Alexa-labeled secondary anti-goat antibody before analysis.

Acknowledgements

This work was supported by grants of the FWF (P16229-B07, SFB-F021). The help from T Guszczynski (NCI), E Rolser, Dr G Quintini and Dr M Schaefer (the latter three from AltanaPharma, Konstanz) for technical assistance, expression of recombinant PKCθ proteins and helpful discussions, respectively, is gratefully acknowledged. All experiments comply with the current laws of Austria.

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