Transient PKCα shuttling to the immunological synapse is governed by DGKζ and regulates L-selectin shedding

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Accepted 5 March 2013

Summary

Considerable evidence indicates that diacylglycerol (DAG) generation at the immunological synapse (IS) determines T cell functions by regulating the duration and amplitude of Ras/ERK signals. The exact mechanism by which DAG regulates Ras/ERK activation downstream of the T cell receptor (TCR) nonetheless remains poorly understood. Here we characterize PKCα as a previously unrecognized component of the machinery that translates cell receptor occupancy into Ras/ERK-propagated signals. We show transient translocation of PKCα to the IS, mediated by DAG generation at the contact area. Diacylglycerol kinase (DGK)ζ negatively regulated PKCα translocation kinetics, whereas PKCα activity limited its own persistence at the IS. Coordinated activation of DGKζ and PKCα in response to antigen recognition regulated the amplitude and duration of Ras/ERK activation; this in turn mediated early processes of T cell surface proteolysis such as L-selectin shedding. Analysis of DGKζ-deficient mice further showed that increased DAG signaling is translated to downstream elements of this pathway, as reflected by enhanced PKCα-dependent L-selectin shedding. We propose that early activation of a DAG–PKCα axis contributes to the mechanisms by which antigen affinity translates into TCR biological responses.

Key words: Diacylglycerol kinase, Immune synapse, Protein kinase C

Introduction

In the adaptive immune response, the fine-tuning of T cell activation is essential to prevent autoimmune disease, inflammation or allergic response, and for some anti-tumor therapies. The ability to manipulate the T cell response is thus a major objective in the clinical treatment of these pathologies. T cell activation is initiated after lymphocyte contact with antigen-presenting cells (APC). Specific antigen recognition by the T cell receptor (TCR) allows rapid hydrolysis of PIP2 (phosphatidylinositol 4,5-bisphosphate), which results in intracellular Ca2+ release and DAG (diacylglycerol) generation. Polarized DAG generation at the IS allows selective activation of a broad range of effectors, including kinases of the PKC and PKD (protein kinase C, D) families, the Ras GEF (guanine nucleotide exchange factor) RasGRP1, and the Rac GAP chimaerin (Almena and Mérida, 2011). Local activation of these proteins initiates a cascade of signals that control the Ras/ERK/AP-1 and CARMA/NFκB pathways, which act in concert with the Ca2+/calcineurin/NFAT pathway to ensure onset of the T cell activation program. Strict control of DAG levels is necessary during IS formation to provide the signal location and intensity needed for a correct immune response. Diacylglycerol kinases (DGK) convert DAG to phosphatidic acid (PA), thus acting as negative regulators of DAG-mediated signals. T lymphocytes express at least two DGK isoforms (ζ and α), of which DGKζ is the major form responsible for DAG metabolism at the IS (Gharbi et al., 2011).

The PKC Ser/Thr protein kinases are principal DAG targets. In mammals, this family is composed of ten members, grouped as classical (cPKC), novel (nPKC) and atypical (aPKC) based on regulatory domain structure and ability to respond to DAG and Ca2+ (Newton, 2010). The PKC localize predominantly in the cytosol of resting cells, where they are catalytically inactive due to autoinhibition by their pseudosubstrate domain. Following receptor activation, PKC isotype-specific signals facilitate membrane translocation, promoting conformational changes that displace the pseudosubstrate moiety from the catalytic cleft and allow phosphorylation of specific substrates. The novel PKCζ is abundant in hematopoietic cells and is the only PKC selectively recruited to the IS through interaction with the costimulatory molecule CD28. PKCζ mediates activation of the CARMA/NFκB axis, leading to enhanced IL-2 production (Huang et al., 2002; Kong et al., 2011; Matsumoto et al., 2005). PKCζ-deficient mice have severe defects in Th2- and Th17-mediated responses and in T regulatory cell development. These mice nonetheless develop a normal protective Th1 immune response, as they are able to clear viral infections, and have intact CD8+ effector memory responses (Hayashi and Altman, 2007; Marsland et al., 2005).
PKCa is proposed to act in concert and/or in alternative pathways with PKCθ to regulate the IkB/NFkB axis (Trushin et al., 2003); it has additional functions including HIV reactivation (Trushin et al., 2005), TCR downregulation (von Essen et al., 2006) and granule exocytosis (Grybko et al., 2007). PKCa-deficient mice have a severe defect in the Th1 response and IFNγ secretion (Pfeifhofer et al., 2006). The phenotype of PKCθ/PKCa double-deficient mice (Gruber et al., 2009), as well as the potent immunosuppressive effect of the dual PKCθ/PKCa inhibitor AEB071 (sotrastaurin) (Baier and Wagner, 2009), further suggest that these two kinases may have non-redundant functions in the regulation of T cell activation.

Here we report rapid, transient PKCa recruitment to the IS during T cell/APC encounter, through a mechanism dependent on DAG generation and kinase activity. We show that DGKζ-mediated DAG consumption regulates local PKCa recruitment at the T cell/APC contact area. Pharmacological and genetic approaches further demonstrate that PKCa is necessary for TCR-mediated early activation of the Ras/ERK axis and the regulation of cell surface protein proteolysis, as measured by L-selectin shedding. Analysis of DGKζ-deficient mice suggests that the role of DGKζ in T cell hyperactivation can be attributed at least in part to PKCa activation. Our results identify PKCa as a component of the DAG-based signalosome induced by antigen recognition, and clarify the mechanisms by which DAG generation at the IS regulates T cell function.

Results
PKCa-GFP localizes at the IS following TCR stimulation
We previously detected PKCa in activated TCR complexes, which suggested that PKCa is recruited to the TCR/APC contact site (Gharbi et al., 2011). To study endogenous PKCa translocation dynamics in response to TCR-mediated signals, we used classical membrane fractionation assays and assessed translocation kinetics in Jurkat T cells stimulated with anti-CD3/CD28 antibodies (Fig. 1A). Membrane localization was detected from the first minute of TCR activation, and then decreased with kinetics that closely resembled that of PLCγ phosphorylation and membrane recruitment. Quantification of plasma membrane recruitment suggested that PKCa translocation kinetics was

![Fig. 1. PKCa translocates to the plasma membrane and the IS in response to TCR activation.](image_url)

(A) Jurkat T cells were stimulated with 5 μg/ml anti-CD3/CD28 antibodies for indicated times or with 200 ng/ml PMA for 30 minutes, and crude membrane fractions isolated. Membrane and cytosolic fractions were probed with specific antibodies; human transferrin receptor (hTfR) and GAPDH were used as markers of membrane and cytosolic fractions, respectively. (B) Normalized plasma membrane recruitment is shown for each time point (normalized to time 0). (C) Videomicroscopy of GFP-fused PKCa and PKCθ constructs transiently expressed in Jurkat T cells during antigen presentation. Raji B cells, CMAC-prestained and pulsed with 1 μg/ml SEE (blue), were used as APC (antigen-presenting cells). APC were added as droplets to adhered T cells to capture initial IS formation, and frames were acquired every 10 seconds for 12.5 minutes; consecutive frames taken every 30 seconds are shown. (D) Accumulation of PKCa (gray box) and PKCθ at the IS was measured as the IS:cytosol fluorescence intensity ratio during the course of time-lapse acquisition (n=6 videos), using ImageJ software (v1.45s). (E) Representative view of PKCa and actin-cherry translocation dynamics to the IS. Videomicroscopy of Jurkat T cells transfected with PKC-GFP and actin-cherry, stimulated 24 hours post-transfection with SEE-loaded APC. Contact between APC and T cell is shown for consecutive frames. Scale bars: 10 μm (C); 2 μm (E).
similar to that of PKCθ; however, the latter showed sustained accumulation at the membrane (Fig. 1B). Complete plasma membrane translocation of PKCα was observed after phorbol 12-myristate 13-acetate (PMA) treatment, which was used as a control.

To study PKCα dynamics after T cell activation, we adopted a T cell stimulation model, using antigen-pulsed Raji B cells as APC, and time-lapse confocal microscopy analysis of T cells expressing PKCθ or PKCα fused to green fluorescent protein (GFP). GFP-PKCθ translocation was rapid, within the first 40–60 seconds after initial cell–cell contact, and the kinase remained at the IS at least 15 minutes after SEE presentation, as reported (Fig. 1C, bottom) (Yokosuka et al., 2008). Whereas non-loaded APC did not promote PKCα relocalization to the T cell–APC contact area (Fig. 1C, top), we observed rapid PKCα-GFP translocation to the IS at 20–40 seconds after initial contact with SEE-loaded APC. Quantification analysis showed that the translocation kinetics of PKCα with SEE-loaded APC. In Jurkat T cells incubated with SEE-loaded Raji B cells were fixed and stained with anti-PKCα antibody (supplementary material Fig. S1A). We measured localization of phosphorylated PKCα in the C-terminal ‘turn motif’ (PKCα-Thr-638) to trace the mature form of PKCα (supplementary material Fig. S1B). We observed pPKCα accumulation at the IS in 43% of the T/B cell conjugates. Thr-638 is thought to be an autophosphorylation site and reflects the activated PKCα form at the IS (Parekh et al., 2000). We also evaluated PKCα localization in primary mouse T cells, using the OTI mouse model of transgenic TCR restricted to MHC class I (Hogquist et al., 1994). As in Jurkat T cells, some conjugates showed PKCα-positive IS, which coincided with accumulation of PKCθ, used as an IS marker (supplementary material Fig. S1C).

**DGKα limits PKCα-GFP translocation to the IS during antigen presentation**

Membrane translocation of classical PKC comprises the initial Ca2+-mediated exposure of the DAG-binding C1 regions, which promote stabilization of these proteins in an active conformation at the membrane (Gallegos and Newton, 2008). To determine the Ca2+ contribution, we examined PKCα translocation to the IS in cells in which Ca2+ influx was prevented by preincubation with the Ca2+ chelator EGTA. Cells were pretreated with EGTA for 30 minutes prior to addition of SEE-loaded APC. In these conditions, PKCα-GFP was unable to translocate to the IS and remained cytosolic during antigen presentation (Fig. 2A,B). To confirm the requirement for Ca2+ generation. We compared the dynamics of PKCα recruitment to the IS with the DAG metabolic rate at this site, using a fluorescent DAG sensor (Carrasco and Merida, 2004) fused to the cherry tag (cherry-C1ab). As reported (Gharbi et al., 2011), the sensor accumulated at the IS after T cell/APC conjugation (Fig. 2C). PKCα-GFP colocalized with cherry-C1ab, although detailed analysis of translocation dynamics showed that the DAG sensor reached the IS more rapidly than PKCα-GFP (Fig. 2D). This indicates that DAG increase at the IS precedes PKCα recruitment.

![Figure 2](image-url)

Fig. 2. PKCα translocation requires Ca2+ and follows DAG production at the IS. (A) PKCα-GFP translocation was monitored as in Fig. 1C, for cells untreated or pretreated with 2 mM EGTA (30 minutes). Representative frames are shown; 0 seconds is the time before T cell and APC contact. (B) Quantification of the PKCα-GFP IS:cytosol fluorescence ratio for each time frame (n=4 videos). (C) Jurkat T cells were co-transfected with PKCα-GFP and the DAG sensor C1ab fused to cherry (cherry-C1ab). Images were captured while APC (blue) initiated contact with T cells. C1ab accumulation at the IS was compared with that of PKCα; consecutive frames are shown from a representative video. (D) A line was drawn and intensities in each channel were represented using the ImageJ RGB profile plot plugin. C1ab (red) and PKCα (green) in contact with the APC (blue). The plot line crossed the site of APC–T cell contact (a.u., arbitrary units). Scale bars: 10 μm (A); 2 μm (C).
We recently reported a critical role for DGKζ in the control of DAG levels at the IS (Gharbi et al., 2011). To determine whether DAG can regulate PKCα localization, we analyzed the consequences of altering DAG metabolism at the IS. PKCα-GFP was co-expressed with cherry (Fig. 3A) or DGKζ fused to cherry (cherry-DGKζ WT) (Fig. 3B) in Jurkat T cells, which were then exposed to SEE-loaded APC. Cherry-DGKζ overexpression did not affect PKCα-GFP IS localization, but reduced PKCα-GFP persistence at the IS by ~70% compared to controls (10–20 seconds versus 60–80 seconds IS retention time) (Fig. 3B–D; supplementary material Movie 2). This effect was due to DAG depletion at the IS, since expression of the kinase-inactive DGKζ mutant fused to cherry (cherry-DGKζKD), which no longer catalyzes DAG phosphorylation into PA (Santos et al., 2002), did not alter PKCα translocation dynamics to the IS (Fig. 3C,D; supplementary material Movie 3).

To further assess the DGKζ contribution, we measured the effect of DGKζ knockdown on PKCα-GFP translocation to the IS (Fig. 3E). Western blot analysis confirmed that both, the long and short DGKζ isoforms were efficiently silenced. Control shRNA-transfected cells conjugated with SEE-pulsed APC showed transient PKCα-GFP translocation to the IS, with kinetics...
similar to that of cells expressing PKCα-GFP alone (Fig. 3F, top; supplementary material Movie 4). In DGKζ knocked-down cells, PKCα-GFP accumulation at the IS was sustained up to 240 seconds from the time of initial cell–cell contact (Fig. 3F,G; supplementary material Movie 5). After this time, PKCα-GFP lost its IS localization. These data suggest that DGKζ negatively regulates PKCα translocation to the IS through local control of DAG metabolism.

PKCα dissociation from the membrane is regulated by enzyme activity

Polarized DAG production is preserved throughout the duration of lymphocyte contact with the APC, whereas PKCα localizes transiently at this site; this suggests that additional mechanisms regulate PKCα dissociation from the IS. Studies of classical PKC show that these isoforms are unstable in their open/active conformation. They have lower affinity for DAG than novel PKC, and are prone to C-terminal autophosphorylation, which dissociation from the membrane is regulated by its own kinase activity.

We analyzed the specific contribution of PKCα to the control of DGKζ-mediated regulation of the Ras/ERK pathway and found that, similar to inhibitor treatment, PKCα knockdown reduced TCR-mediated MEK hyperphosphorylation in DGKζ-depleted cells (Fig. 5B). The effect of PKCα depletion on pMEK was not complete, which might be attributed to incomplete PKCα knockdown or to additional components that contribute to DGKζ-mediated control of this pathway requires cPKC activity (Fig. 5A). We previously reported that TCR signals induce a DGKζ mobility shift, representing putative post-translational modification. Here we observed that cPKC inhibitor treatment reduced this shift, which was more pronounced in the short isoform, suggesting cPKC-mediated regulation of DGKζ.

PKCα regulates TCR-mediated Ras/ERK activation

DGKζ has been found to act as a negative regulator of the Ras/ERK pathway, that is triggered after TCR activation (Zhong et al., 2003). To assess PKCα input in DGKζ-regulated events, we next monitored this signaling axis during anti-CD3/CD28 costimulation in DGKζ-depleted cells. As predicted, DGKζ knockdown induced Ras pathway hyperactivation as shown by MEK and ERK1/2 hyperphosphorylation. This effect was reduced by pharmacological inhibition with Gö6976, indicating that DGKζ-mediated control of this pathway requires cPKC activity (Fig. 5A). We previously reported that TCR signals induce a DGKζ mobility shift, representing putative post-translational modification. Here we observed that cPKC inhibitor treatment reduced this shift, which was more pronounced in the short isoform, suggesting cPKC-mediated regulation of DGKζ.

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![Fig. 4. PKCα IS persistence is regulated by its own kinase activity.](image-url)

(A) Live images of PKCα-GFP localization in Jurkat T cells pretreated with classical or novel PKC inhibitors (100 nM BIM, 100 nM Gö6976, or 6 μM rottlerin) 30 minutes prior to addition of SEE-loaded APC (blue). Merged images are shown from representative videos for each condition. (B) PKCα-GFP localization was evaluated after 30 minutes incubation with SEE-loaded Raji B cells (blue) in inhibitor-treated T cells as in A. Representative images are shown. (C) PKCα-GFP IS localization was quantified at 30 minutes after addition of SEE-loaded APC (mean ± s.e.m.; n=15 images). Unpaired t test with Welch’s correction; ***P=0.0002; ns, not significant. Scale bars: 5 μm (A); 10 μm (B).
activation was more pronounced in cells stimulated with anti-CD3 compared to anti-CD3/CD28, showing a twofold increase over siRNA control (Fig. 5C). This hyperactivation was lost in DGKζ/PKCζ double-silenced cells thus showing sensitivity to PKCζ expression (Fig. 5C). These data highlight the important role of PKCζ downstream of the TCR and demonstrate that, in the absence of DGKζ, a greater DAG accumulation upon TCR activation promotes a PKC-driven response.

DGKζ is proposed to negatively regulate the Ras/ERK pathway by modulating DAG-dependent activation of the Ras GEF RasGRP1 (Zhong et al., 2003). To further determine the impact of PKCζ on Ras regulation, we monitored Ras activation in DGKζ or PKCζ-silenced cells. We performed Ras-GTP pull-down assays and observed increased TCR-triggered Ras-GTP loading of DGKζ-depleted cells (Fig. 5D) after stimulation with anti-CD3 antibodies, in agreement with previous reports (Zhong et al., 2003). PKCζ knockdown blocked the gain of function effect of DGKζ on Ras activation suggesting that this kinase acts upstream of Ras in a DAG-sensitive manner.

**PKCζ regulation of the Ras/ERK pathway contributes to CD62L shedding**

Activation of the Ras/ERK pathway lies at the core of several events critical for the correct onset of the T cell activation
program. We tested the functional consequences of PKCζ in primary T lymphocytes by focusing on the expression of two well-characterized ERK effectors, the e-type lectin (CD69) and L-selectin (CD62L). CD69 is a bona fide Ras/ERK/AP-1 activation marker that is transcriptionally upregulated shortly after TCR triggering (Sancho et al., 2005). CD62L is strongly expressed on the naïve T cell surface; these levels decrease sharply after activation due to TCR-triggered ERK-mediated shedding (Sinclair et al., 2008). CD62L down- and CD69 upregulation are two hallmarks of T cell activation that take place shortly after antigen encounter and correlate closely with the antigen affinity threshold (Moreau et al., 2012).

Mouse splenocytes were pretreated with Gö6976 or rottlerin to inhibit cPKC or nPKC respectively, then stimulated with anti-CD3 antibody; CD62L and CD69 levels were measured by flow cytometry (Fig. 6A,B). Both inhibitors reduced CD69 expression, which was more notable after Gö6976 treatment. Rottlerin however did not prevent CD62L loss, in contrast with the near-total blockade of CD62L shedding observed after cPKC inhibition. A detailed kinetic analysis confirmed that this effect was similar in both CD4+ and CD8+ T cells (Fig. 6B).

Phosphorylation of protein kinase D (PKD), which lies downstream of PKCζ and PKCθ (Spitaler et al., 2006), was used as a control of both PKC inhibitors in western blot. Analysis confirmed the Gö6976 effect on TCR-triggered Ras/ERK pathway activation, as tested by ERK phosphorylation after long-term anti-CD3 treatment (supplementary material Fig. S2A); in these conditions, rottlerin had a milder effect on pERK induction. As reported (Sinclair et al., 2008), inhibition of ERK activation with the MEK1 inhibitor (PD98059) prevented CD3-induced CD62L shedding (supplementary material Fig. S2B); in these conditions, rottlerin had a milder effect on ERK activation with the MEK1 inhibitor (PD98059) prevented CD3-induced CD62L shedding (supplementary material Fig. S2B), confirming that this process is regulated by cPKC and that it requires ERK activation for an adequate response. These data corroborate our results with the human T cell line and indicate a role for this isoform in TCR-mediated CD62L shedding.

Discussion

Participation of PKCζ in the interplay of synapse regulation and early T cell response had been previously ruled out; the sole PKC isoform found to accumulate stably at the IS was the novel PKCθ, which thus constituted an important IS marker (Tseng et al., 2008; Yokosuka et al., 2008). Based on observations from several experimental models, we report here the direct recruitment of PKCζ to the IS during T cell activation. This relocation was both rapid and transient, in contrast to the sustained accumulation of PKCθ. The rapid kinetics of PKCζ translocation suggests a role for this protein in membrane-linked events early after TCR triggering. In agreement with this observation, we characterized PKCζ as a component of the Ras/ERK axis, and demonstrate its contribution to TCR-mediated CD62L shedding.

We observed that in response to Ca2+ elevation, PKCζ moves to the actin cytoskeleton-rich T cell/APC contact area, where it senses DAG. Pharmacological inhibitors of cPKC did not block PKCζ translocation, but impaired its return to cytosol. This mechanism of action is consistent with studies showing that cPKC autophosphorylation at conserved C-terminal residues promotes membrane dissociation and autoinhibition by pseudosubstrate intramolecular binding (Newton, 2003). Our data are also compatible with models in non-hematopoietic cell systems in which PKCζ oscillates between active/open and inactive/semi-open conformations, in an equilibrium governed by DAG binding and autophosphorylation. Our findings, consistent with those of previous studies, provide a better understanding of the differences between PKCζ and PKCθ with respect to the signals that regulate their recruitment to the IS. Whereas PKCζ senses TCR-triggered Ca2+ and DAG generation at the IS, PKCθ not only has greater affinity for DAG, but is also recruited to the IS through a protein–protein interaction between its V3 domain and CD28 (Kong et al., 2011); this explains why PKCθ is more stable and easily detectable at the IS. The difference in kinetics between these two isoforms also helps to explain their distinct substrate specificity, with PKCζ acting early and sensitive to low TCR signals, whereas PKCθ controls sustained events coordinated by CD28. This mode of action correlates well with studies that showed PKCζ participation in early CD3/CD28-mediated IκB kinase stimulation, followed by a later, PKCθ-dependent activation phase (Trushin et al., 2003). Our data show that Ras/ERK activation in response to TCR triggering was more sensitive to PKCζ depletion, whereas co-stimulatory signals are less dependent on PKCζ. We can foresee that the stimulation intensity could potentiate the specific activation of the classical or the novel PKC, determining the fate of the response.

Studies in non-lymphoid cells have identified DGKζ as a negative regulator of PKCζ, which in turn modulates DGKζ activation by phosphorylation at its MARCKS (myristoylated alanine-rich C-kinase substrate) region (Luo et al., 2003), a cluster of basic and serine residues analogous to those in the MARCKS protein. A similar mechanism appears to operate at the IS, where DGKζ and PKCζ each regulates the other. [Ref: 2182 Journal of Cell Science 126 (10) 2003]
Fig. 6. Functional effect of PKCα on the regulation of L-selectin shedding during the early T cell response. (A,B) Splenocytes from wild-type (WT) mice were pretreated with 0.1% (v/v) DMSO (vehicle), 100 nM Gö6976 or 6 μM rottlerin, and stimulated with 5 μg/ml anti-CD3 antibody. Surface expression of CD62L and CD69 was analyzed by flow cytometry. (A) Histograms show CD62L and CD69 expression in CD4+ and CD8+ T cells at 4 hours post-stimulation for each treatment. (B) Relative cell surface CD62L expression (left) and the percentage of CD69 expression (right) in CD4+ and CD8+ T cells were quantified at each time point (mean ± s.e.m.; n=4 mice). (C,D) Splenocytes from WT and DGKγ-deficient (DGKγ−/−) mice were stimulated with 2.5 μg/ml anti-CD3 antibody. Cell surface expression of CD62L and CD69 induction are shown. (C) Overlays from representative WT and DGKγ−/− mice in CD8+ and CD4+ T cells at 30 minutes post stimulation are shown (top). Relative expression in CD8+ T cells (bottom) was quantified as in B (mean ± s.d.; n=4 mice/phenotype). (D) CD69 induction at 4 hours post-stimulation with 2.5 μg/ml anti-CD3 antibody (representative overlay shown, top). Relative CD69 mean fluorescent intensity (MFI) was quantified in CD8+ T cells from WT and DGKγ−/− deficient splenocytes (bottom); mean ± s.e.m.; n=4 mice, (P=0.3429). (E,F) Splenocytes from WT and DGKγ−/− deficient mice were pretreated with PKC inhibitors as in A and stimulated with 2.5 μg/ml anti-CD3 antibody for 0–240 minutes. (E) Overlays are shown of cell surface expression of CD62L in CD8+ T cells. (F) Relative cell surface expression of CD62L was quantified (mean ± s.e.m.; n=4 mice).
Phosphorylation of the MARCKS domain seems necessary for DGKζ targeting to the plasma membrane in lymphoid (Santos et al., 2002) and non-lymphoid cells (Abramovici et al., 2003). DGKζ Ser-to-Ala MARCKS domain mutants neither metabolize DAG at the IS nor prevent TCR-induced IL2 transcription (Gharbi et al., 2011). We observed a direct correlation between TCR activation and a slow-migrating DGKζ band, which probably reflects post-translational modification; this effect was sensitive to PKC inhibition, as the molecular weight shift was reduced after G66976 co-treatment and PKCa downregulation. The previous reports combined with our findings here suggest concerted cross-regulation of PKCζ and DGKζ, to guarantee an adequate Ras-ERK activation threshold in response to antigen presentation.

In T lymphocytes, conclusive evidence has shown the negative role for DGKζ in DAG-mediated regulation of the RasGRP1/Ras axis (Roose et al., 2005). The RasGTP generated after RasGRP1 activation binds to the Ras allosteric pocket in SOS (son of sevenless), establishing a positive feedback loop for Ras activation. RasGRP1 and SOS pathways are proposed as analog and digital determinants in the regulation of Ras activation in T lymphocytes (Das et al., 2009). The hyperactive Ras/ERK phenotype of DGKζ-deficient mice concurs with this model, although recent experimental results suggest that the regulatory mechanism of this axis via DGKζ is more complex (Riese et al., 2011). This disparity implies that DGKζ deficiency has effects other than merely promoting increased DAG/RasGRP interaction, and indicates our participation of additional DAG-regulated proteins. Our data identify PKCa as a DAG/DGKζ-regulated molecule and show its contribution to Ras GTP loading. These findings correlate with studies that describe a role for PKCα in PKCα-mediated phosphorylation, which activates regulation of Ras activation through direct RasGRP1 phosphorylation. The possible regulation of RasGRP1 (which bears a DAG-binding domain and Ca2+-regulated EF hands) by the Ca2+-regulated PKCa might increase Ras/ERK pathway responsiveness to TCR-triggered PLC activation. RasGRP3 phosphorylation by PKCβ2 (Teixeira et al., 2003) and regulation of Ca2+-dependent ERK activation by RasGRP/PKCδ and the Ca2+ sensor STIM1 in B cells are clear examples of this type of crosstalk (Limnander et al., 2011).

The identification of PKCa as a component of the Ras/ERK pathway correlates with a central role for this isoform in the rapid shedding of CD62L and the upregulation of CD69 that take place after TCR triggering. CD62L shedding is mediated primarily by the metalloprotease TNFα-converting enzyme (TACE/ADAM17), although the precise mechanism governing TACE-mediated CD62L shedding is not fully characterized. In addition to PKCa contribution to Ras/ERK regulation, a more direct PKCa effect on CD62L shedding correlates with previous reports that implicate PKC in the regulation of TACE-mediated shedding processes (Killock and Ivetić, 2010; Kveiborg et al., 2011) and PKCa interaction with the CD62L cytoplasmic tail after anti-CD3 stimulation (Kilian et al., 2004).

CD62L cleavage ensures exit of activated T cells from lymph nodes and their migration to non-lymphoid organs (Arbouet et al., 1994). This process was initially thought essential to prevent reentry of active T cells into lymph nodes (Galkina et al., 2003; Venturi et al., 2003). Nonetheless, CD8+ memory cells from mice transgenic for a shedding-resistant CD62L home normally to infection sites, although their virus-clearing ability is compromised (Richards et al., 2008). A role for CD62L ectodomain proteolysis in promoting viral clearance also correlates with recent reports showing that a CD62L shedding-resistant mutant impairs CD8+ cell lytic activity following antigen recognition by tumor antigen-reactive T cells (Yang et al., 2011). Overall, these studies suggest a biochemical link between antigen-induced loss of CD62L and CD8+ effector functions.

Our identification of PKCa at the IS and its participation in TCR-mediated signaling integrates this isoform as a component of the DAG signalosome, critical for optimal onset of the T cell activation program. Implication of the DGKζ/PKCα/ERK axis in the regulation of antigen-mediated CD62L shedding, itself linked to virus clearance and anti-tumor immunity, is also consistent with the strong antiviral and anti-tumor activity in DGKζ-deficient mice (Zhong et al., 2003; Riese et al., 2011). Our study raises important questions that remain to be addressed, including the nature of the mechanisms that mediate PKCa regulation of RasGRP1/ERK, and the effect of this axis on TACE-mediated shedding of other surface molecules. Broad PKC inhibitors are being explored for manipulation of the immune response, and are in clinical trials to promote immunosuppression for organ transplant. It is thus essential to better define the impact of PKC isoforms during the immune response, to complement our current understanding of immune surveillance and to improve its therapeutic modulation.

Materials and Methods

Reagents and antibodies

Superantigen Staphylococcus enterotoxin E (SEE) was from Toxin Technology (Sarasota, FL); the cell tracker chloromethyl derivative of aminocoumarin (CMAC) and Rhodamine Phalloidin were from Molecular Probes (Life Technologies, Leiden, The Netherlands). Anti-human and -mouse CD3-ε (anti-CD3) and CD28 antibodies were from BD PharMingen (San Diego, CA), biotinylated secondary antibody was from Jackson ImmunoResearch (West Grove, PA), pPKCζ, pPKD, pPLCγ, pPKCaζ-638 from Cell Signaling Technology (Beverly, MA), monoclonal anti-PKCα antibody from BD Biosciences (San Diego, CA) and anti-PKCα from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-DGKζ was from Abcam (Cambridge, UK) and anti-Tfr antibody from R&D Systems (Minneapolis, MN).

Cell lines and culture conditions

Human leukemia Jurkat T cells were maintained in DMEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Sigma, St Louis, MO) and 2 mM L-glutamine (BioWhittaker). Raji human B lymphoma cells were cultured in RPMI 1640 (BioWhittaker) supplemented as above. Both cell lines were cultured at 37°C in a 95% humidified atmosphere with 5% CO2 and maintained at subconfluence (<5x105 cells/ml).

Mice

Mice were maintained in pathogen-free conditions and handled in accordance with Spanish and European directives; experiments were approved by the Ethics Committee of the Centro Nacional de Biotecnología/CSIC. OTI and DGKζ- mice are described elsewhere (Houquist et al., 1994; Zhong et al., 2003). Experiments were carried out with 3- to 6-week-old mice; WT/DGKζ-deficient mice were littermates.

 Constructs and transient transfection

PKCa fused to green fluorescent protein (GFP) was a kind gift from Dr S. Corbalán-García (University of Murcia, Spain) and is reported elsewhere (Bolosver et al., 2003). Myc-tagged DGKζ fused to red fluorescent protein (RFP) or cherry, as well as C1ab-cherry and actin-cherry have been reported (Gharbi et al., 2011). Jurkat T cell logarthmic growth were transfected as described (Gharbi et al., 2011); briefly, 1.2x106 cells were transfected with 25 μg plasmid DNA by electroporation using a Gene Pulser (BioRad; 270 V, 975 μF). Assays were generally performed 24 or 48 hours post-transfection.
RNA interference of DGKβ and PKCα

pSUPER plasmids (Oligogene) containing the interfering 21 nucleotide sequence of DGKβ human (shRNA DGKβ) or mouse (shRNA DGKβα) control in a hairpin structure were generated in our laboratory (Avila-Flores et al., 2005; Rincon et al., 2011). shRNA oligonucleotides used to silence PKCα have been described (Lone et al., 2010) and shRNA to silence DGKβ corresponds to the same region as the shRNA to DGKβα. Jurkat T cells in logarithmic growth were transfected with 25 μg plasmid DNA or with siRNA oligonucleotides by electroporation as above. In overexpression experiments, PKCα-GFP was electroporated 48 hours after RNA interference (RNAi). Cells were assayed by time-lapse microscopy at 72–96 hours post-RNA treatment.

Anti-CD3 and anti-CD3/CD28 stimulation

For TCR stimulation, Jurkat T cells in logarithmic growth were pooled (106 cells in 1 ml of medium/condition) and held on ice (10 minutes). Cells were stimulated with soluble anti-CD3 or anti-CD3/CD28 antibodies (1 μg/ml) for indicated times and lysed (1% Igepal, 150 mM NaCl, 20 mM HEPES, pH 7.4, supplemented with protease and phosphatase inhibitors sodium orthovanadate, β-glycerophosphate, phenylmethlysulfonil fluoride, leupeptin and aprotonin) for western blot of total cell extracts. Where indicated, cells were pretreated for pharmacological inhibition with bisindolylmaleimide (BIM) (100 μM), G66976 (100 nM), rottlerin (6 μM) or PD98059 (50 μM or 2 μM) (37°C, 30 minutes) prior to stimulation (all from Calbiochem, MERCK–Millipore, Darmstadt, Germany).

Membrane fractionation

Jurkat T cells were fractionated as described (Merino et al., 2008). Cells were incubated on ice with 5 μg/ml anti-CD3 alone or combined with 5 μg/ml anti-CD28 antibody (30 minutes). Secondary crosslinking antibody (20 μg/ml) was added to anti-CD3/CD28-stained cells (10 minutes, on ice), and transfected to 37°C during time course of stimulation. Cells were lysed in digitonin lysis buffer (40 μg/ml digitonin, 5 mM Tris-HCl pH 7.5, 10 mM NaCl, 0.5 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol (DTT), supplemented with protease and phosphatase inhibitors) for 15 minutes on ice. Cytosol was collected after centrifugation (4500 g, 4 minutes, 4°C) and pellets were further solubilized in NP-40 lysis buffer (0.2% Igepal, 5 mM Tris-HCl pH 7.5, 10 mM NaCl, 0.5 mM MgCl2, 1 mM EGTA, 1 mM DTT) for 10 minutes on ice. Membrane fractions were separated from insoluble fractions by centrifugation (15,000g, 15 minutes, 4°C). Each fraction was quantified by Bradford assay and analyzed by western blot.

Ras pull-down assays

Jurkat T cells were transfected with interfering nucleotides and assayed 72 hours post-RNA treatment; 3×106 cells/condition were stimulated with 1 μg/ml anti-CD3 (5 minutes). Cells were pelleted, resuspended in 150 μl Mg-containing buffer (MLB: 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal, 10 mM MgCl2, 1 mM EDTA, 2% glycerol) supplemented with protease inhibitors and lysed by 15 passages through a 30-gauge needle. The suspension was cleared by centrifugation (13,000g, 15 minutes, 4°C) and the supernatant pre-cleared with glutathione S-transferase (GST)-agrose beads (10 minutes, 4°C). Lysate (150 μg) was incubated (30 minutes) with 10 μl GST-Raf 1-Ras-binding domain beads (GST-Raf1-RBD; Upstate Biotechnology, Lake Placid, NY). Beads were washed three times with MLB buffer and eluted by boiling in SDS-PAGE loading buffer. Eluted proteins were analyzed by immunoblot using a pan-Ras mAb (Oncogene) to detect bound Ras.

Immunostaining

T cells (2.5×106 cells) transfected or at 48 hours post-transfection with PKCα-GFP, were transfected to pohDL-lysine-coated coverslips (10 minutes, on ice). Where indicated, cells were pretreated with 100 nM BIM (15 minutes, 37°C). Raji B cells (2×106 cells/ml) were stained with 50 μM CMAC and incubated alone or with 1 μg/ml SEE (30 minutes, 37°C), then added as droplets at a 1:1 ratio to Jurkat T cells on ice to capture early reaction kinetics. Jurkat T cells/APC were incubated on ice at 37°C (5 minutes). Immunostaining was performed as described (Cernuda-Morollón et al., 2010). Cells were then fixed (4% paraformaldehyde), washed and permeabilized (5 minutes, on ice) in 0.2% Triton/PBS, then blocked in 5% BSA/PBS. Specific antibodies were used as stated in figure legends (30 minutes, 37°C). For experiments with primary T lymphocytes, spleen cells from OTI mice were presented with the EL4 mouse lymphoma cell line, loaded with the OVA peptide and pre-stained with 50 μM CMAC. CTL/EL4 were incubated (10 minutes, 37°C). Mounted slides were analyzed by confocal microscopy (LSM-510 META, Zeiss) and images processed with ImageJ and Adobe Photoshop software.

Time-lapse confocal microscopy

Live Jurkat cells were imaged as described, with minor modifications (Gharbi et al., 2011). Briefly, at 48 or 72 hours post-transfection, cells were collected (0.5×106 cells/ml) in HBSS (25 mM HEPES–KOH pH 7.4, 1 mM MgCl2, 1 mM CaCl2, 132 mM NaCl, 0.1% BSA) supplemented with 2% FBS. Cells were transferred to poly-lysine-coated glass chambers (Nunc, Lab-Tek, Rochester, NY) and placed on a 37°C stage of a laser-scanning confocal microscope (FV1000, Olympus). Images were collected every 10 seconds before, during and after addition of control or SEE-pulsed Raji B cells stained with CMAC (added as droplets from 2×107 cells/ml in HBBS). Videos were analyzed using image analysis software (ImageJ v1.45s). PKCα-GFP time of translocation was quantified by measuring the accumulation time of GFP-positive signals at the IS in each condition tested. Data were averaged from at least 15 videos per condition. For fluorescence IS signal quantification, GFP–fluorescence intensity ratios at the IS versus the cytosol were measured using an ImageJ plugin developed in-house (C. Sorzano, CNB/CSIC) (Rincón et al., 2011).

Primary splenocyte stimulation and analysis of cell surface receptors

T cells were isolated from spleen, collected in RPMI (5% L-glutamine, 10% FBS) and maintained at 37°C in 5% CO2 throughout the assays. Cells (2×106 cells/ml) pretreated with specific inhibitors or vehicle were stimulated with 5 or 2.5 μg/ml anti-CD3 antibodies for up to 4 hours. Cells were collected in ice-cold PBS and surface-stained for FACS analysis or western blot. For flow cytometry, cell surface receptors were stained with saturating concentrations of fluorophore-conjugated antibodies (CD4–APC, CD8–PerC, CD62L–FITC, CD69–PE, from BD Pharmingen or eBioscience, San Diego, CA) in PBS staining buffer (0.5% BSA/PBS) (30 minutes, 4°C). Cells were washed, fixed in 0.2% paraformaldehyde/0.5% BSA/PBS, and held at 4°C for flow cytometry using Galillos and Cytomix FC500 cytometers (Beckman Coulter Inc.). Live cells were gated using forward and side scatter parameters. Each sample was acquired for a minimum of 70,000 events and data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Acknowledgements

The authors thank laboratory colleagues for helpful discussion, Drs J. Millán, Y. Carrasco and M. Valés-Gómez for feedback on the manuscript, and C. Mark for excellent editorial support. The authors thank Dr S. Corbalán for providing the PKCα-GFP construct. The authors declare no competing financial interests.

Author contributions

S.I.G. and I.M. designed the experiments and wrote the manuscript; S.I.G., J.P.A. and I.M. analyzed and interpreted the data; S.I.G. and A.O. performed the confocal/time-lapse microscopy work; A.A.-F. made the RNAi constructs, designed and performed the membrane fractionation assays and the Ras/MEK/ERK signaling assays; S.I.G. and D.S. performed and analyzed the cell sorting data; G.A.K. supplied the DGKβ-deficient mice and provided input on the manuscript.

Funding

S.I.G. was funded by the JAE-Doc2009/CSIC-FSE program and the Madrid regional government (Centrosome CAM) [grant number S2010/BMD-2305]. A.A.-F. is supported by the Spanish Anti-Cancer Association (AECC), and D.S. holds an FPI fellowship from the Spanish Ministry of Economy and Competitiveness. This work was supported in part by grants from the Spanish Ministry of Health (Instituto de Salud Carlos III) [grant number RD067002071035], the Spanish Ministry of Education [grant number BFU2010-599 21138] and the Madrid regional government (Inmunothercam) [grant number S-SAL-0311] to I.M.

Supplementary material available online at http://jcscbiologists.org/lookup/suppl/doi:10.1242/jcs.118513/-/DC1

References


J. Exp. Med. 201, 2932-2942.


Fig. S1. Immunofluorescence analysis of endogenous PKC\(\alpha\) during antigen presentation with SEE-loaded Raji B cells (APC; blue). (A, B) Jurkat T cells were incubated onto poly-Lysine-coated slides for 10 min. They were then placed on ice and CMAC-stained/SEE loaded APCs (star) were added as droplets, to allow cells to adhere to the coverslips, without initiation of antigen presentation. T–B cells were then incubated at 37°C, 5% CO\(_2\) incubator for 5 min and fixed with paraformaldehyde. Permeabilized cells were probed with PKC\(\alpha\) (A) or pPKC\(\alpha\) (Thr-638) (B) antibodies. Polymerized actin and PKC\(\theta\) were used as markers of T/B cell conjugate with Rhodamin Phalloïdin and anti-PKC\(\theta\) antibody. Representative images are shown for PKC\(\alpha\) staining, and pPKC\(\alpha\) staining. In cells transfected with PKC\(\theta\)-GFP (B), we detected PKC\(\alpha\)/PKC\(\theta\) co-localization, suggesting that both enzymes are not exclusive. (C) Splenocytes from OT-I mice were incubated for 10 min with EL-4 T cells loaded with the peptide OVA antigen (star). Cells were fixed and stained with antibodies to PKC\(\alpha\), PKC\(\theta\) and polymerized actin (Rhodamin Phalloïdin), as in panel A. Scale bars: 5 \(\mu\)m (A); 10 \(\mu\)m (B).
Fig. S2. Rate of CD62L shedding in DGKζ-deficient mice in response to anti-CD3 stimulation. (A) Primary splenocytes from mice WT pre-treated with vehicle or PKC inhibitors (100 nM Gö6976 or 6 μM rottlerin for 30 min before anti-CD3 stimulation), were stimulated with 5 μg/ml anti-CD3 antibody for 0, 30, 60 or 240 min, as in Fig. 6A,B. Cells were lysed for western blotting and pERK and pPKD induction was measured as treatment controls. (B) Primary splenocytes from mice WT were pre-treated with Gö6976 and the MEK inhibitor PD 98059 for 30 min prior to stimulation with 2.5 μg/ml anti-CD3ε, for 0, 30, 60 or 240 min. Two inhibitor doses of PD 98059 were tested: 2 μM (PD1) and 50 μM (PD2). (C, D) Splenocytes from WT or DGKζ-depleted (DGKζ−/−) mice were stimulated with 5 μg/ml anti-CD3 antibody and surface-stained for CD62L (C) and CD69 (D). (E) Splenocytes from WT or DGKζ−/− mice were stimulated with low anti-CD3 concentration (2.5 μg/ml), pre-treated or not with PKC inhibitors, as in Fig. 6E,F. Quantification of CD69 induction is shown from at least three independent experiments (mean±s.d.; n≥4 mice).
Movie 1. Time-lapse videomicroscopy that shows PKCα-GFP (green) and actin-cherry translocation after antigen presentation by SEE-pulsed B cells (blue).

Movie 2. Time-lapse videomicroscopy of the PKCα-GFP (green) IS translocation in cells overexpressing GFP-DGKζWT fused to cherry tag.

Movie 3. Time-lapse videomicroscopy of the PKCα-GFP (green) IS translocation in cells overexpressing the kinase inactive mutant GFP-DGKζKD fused to cherry tag.
Movie 4. Time-lapse videomicroscopy of PKCa-GFP (green) IS localization in Jurkat T cells treated with RNAi control after antigen presentation by SEE-pulsed B cells (blue).

Movie 5. Time-lapse videomicroscopy of PKCa-GFP (green) IS localization in Jurkat T cells knocked down for DGKζ after antigen presentation by SEE-pulsed B cells (blue).

Movie 6. Time-lapse videomicroscopy of Jurkat T cells transiently expressing PKCa-GFP (green) pre-treated with 100 nM of the PKC inhibitor BIM prior to antigen presentation with SEE-loaded Raji B cells (blue).