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Diacylglycerol and Protein Kinase D Localization during T Lymphocyte Activation

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Summary

The serine kinase protein kinase D (PKD) has a cysteine-rich domain (CRD) that binds diacylglycerol (DAG) with high affinity. PKD is cytosolic in unstimulated T cells, but it rapidly polarizes to the immunological synapse in response to antigen/antigen presenting cells (APCs). PKD repositioning is determined by the accumulation of DAG at the immunological synapse and changes in DAG accessibility of the PKD-CRD. Unstimulated T cells are shown to have a uniform distribution of DAG at the plasma membrane, whereas after T cell activation, a gradient of DAG is created with a persistent focus of DAG at the center of the synapse. PKD is only transiently associated with the immune synapse, indicating a fine tuning of PKD responsiveness to DAG by additional regulatory mechanisms. These results reveal the immune synapse as a focal point for DAG and PKD as an immediate and dynamic DAG effector during T cell activation.

Introduction

T lymphocyte activation is initiated by triggering of the T cell antigen receptor (TCR) by peptides presented on major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APC). Engagement of the TCR stimulates a network of signal transduction molecules that control T cell proliferation and differentiation (Kane et al., 2000; van Leeuwen and Samelson, 1999). Key signaling pathways in T cells are regulated by the products of inositol lipid metabolism. For example, antigen receptors stimulate the accumulation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns-(3,4,5)-P_3), a high-affinity docking site for pleckstrin homology (PH) domains in a large variety of signaling molecules (Costello et al., 2002; Harriague and Bismuth, 2002). The TCR also induces phospholipase C (PLC)-mediated hydrolysis of PtdIns-(4,5)-P_2 to produce inositol polyphosphates that regulate intracellular calcium (van Leeuwen and Samelson, 1999). PtdIns-(4,5)-P_2 hydrolysis also generates diacylglycerol (DAG), which binds with high affinity to conserved cysteine-rich C1 domains (for a review, see Kazanietz, 2002; Spitaler and Cantrell, 2004). DAG binding proteins include serine/threonine kinases of the protein kinase C (PKC) and protein kinase D (PKD) families, the Ras guanyl releasing protein (GRP) family of nucleotide exchange factors, and DAG kinases (DGKs) (Carrasco and Merida, 2004; Ebinu et al., 2000; Matthews et al., 200b; Sanjuan et al., 2003; Spitaler and Cantrell, 2004; Tan and Parker, 2003; Wood et al., 2005; Zhong et al., 2003).

One DAG binding protein regulated by the TCR is the serine/threonine kinase protein kinase D (PKD-1), which binds DAG through its N-terminal cysteine-rich domain (CRD) comprising two tandem C1 domains (Marklund et al., 2003; Matthews et al., 2000a, 2000b; Rozengurt et al., 2005; Wood et al., 2005). PKD-1 regulates thymocyte development (Marklund et al., 2003) and regulates the activity of β1 integrins via regulation of the Rap1 GTPase (Medeiros et al., 2005). PKD-1 also modulates class IIa histone deacetylase (HDAC) phosphorylation in lymphocytes (Dequiedt et al., 2005; Parra et al., 2005; Vega et al., 2004). DT40 B cells coexpress two PKD isoforms, PKD-1 and PKD-3, and these are redundant for HDAC regulation (Matthews et al., 2006). One unexplored area of PKD-1 regulation in T cells is the localization of this enzyme as T cells respond physiologically to immune stimulation. This is an important issue because PKDs have different patterns of subcellular localization in different cell types, and PKD-1 and -3 can differentially localize in the same cell (Rey et al., 2003). For example, in HeLa cells, PKD-1 is found in the trans-Golgi network (Maeda et al., 2001; Prestle et al., 1996), where it regulates anterograde membrane trafficking (Prigozhina and Waterman-Storer, 2004; Yeaman et al., 2004). In contrast, in quiescent B lymphocytes, mast cells, and fibroblasts, PKD-1 is cytosolic, but it rapidly translocates to the plasma membrane in response to stimulation with agonists that activate PLCs to produce DAG by PtdIns-(4,5)-P_2 hydrolysis (Matthews et al., 2000a; Rozengurt et al., 2005). This membrane translocation can be transient (Matthews et al., 2000a; Rey et al., 2001a, 2001b) or prolonged (Oancea et al., 2003), depending on cell type and stimulus. The distribution of PKD-1 between the membrane and the cytosol has major consequences for the function of the enzyme. This has been demonstrated directly by experiments comparing the actions of membrane-targeted or cytosolic PKD in thymocytes of transgenic mice (Marklund et al., 2003). Accordingly, because PKD-1 can have divergent functions at different intracellular sites, understanding the mechanisms regulating PKD intracellular positioning is fundamental to understanding PKD function in lymphocyte activation.

A key determinant for PKD localization is DAG binding to the CRD (Maeda et al., 2001; Rey and Rozengurt, 2001). DAG binding is required for PKD activation in lymphocytes (Wood et al., 2005), but the only information about PKD localization in T cells is that PKD can transiently move to the plasma membrane in T cells activated by CD3 antibodies (Medeiros et al., 2005). It is not known where PKD is positioned in T cells activated physiologically by antigen/APCs. In this latter scenario, TCR triggering is a polarized response initiated at the point of contact between the T cell and APC (Davis and Dustin, 2004; Kupfer and Kupfer, 2003). Whether

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PKD signals in the T cell-APC contact zone is difficult to predict, as there is little known of where DAG, the signaling switch for PKD, is localized in T cells responding to immune stimulation. In this context, T cells form a tight contact with antigen-primed APCs, a structure known as the immunological synapse (Bromley et al., 2001; Grakoui et al., 1999; Monks et al., 1998). This is a highly ordered structure characterized by the segregation of receptors and signaling molecules into distinct zones known as supramolecular activation clusters, or SMACs. In stable synapses, these zones are often arranged in concentric areas, with the TCR accumulating in the center, or the cSMAC, whereas integrins are segregated into the periphery, or the pSMAC (Friedl et al., 2005; Monks et al., 1998). In the context of DAG signal transduction, the immune synapse is not a priori the focus for DAG binding proteins: of several DAG binding PKC isoforms expressed in T cells, only PKCθ accumulates at the cSMAC during T cell activation (Kupfer and Kupfer, 2003; Monks et al., 1997). Moreover, Ras GRP1, which binds DAG with high affinity, translocates to Golgi membranes rather than the plasma membrane in activated T cells (Bivona et al., 2003). The failure of DAG binding proteins to colocalize during T cell activation may reflect differences in the selectivity and affinity of different CRDs for different species of DAG (Madani et al., 2001). Also, localization of DAG binding proteins is influenced by protein-protein interactions. For example, cSMAC translocation of PKCθ is coregulated by DAG and Vav-1 (Diaz-Flores et al., 2003; Villalba et al., 2002). It is thus not known if the immunological synapse plays any role as a site for DAG/PKD signaling in T cells.

One way to visualize the spatial distribution of lipid second messengers is to use enhanced green fluorescent protein (EGFP)-tagged lipid binding domains as fluorescent indicators in living cells. This technology has been used to show that antigen receptors stimulate rapid and sustained accumulation of PtdIns-(3,4,5)-P_3 (Costello et al., 2002; Harriague and Bismuth, 2002), but that this lipid localizes relatively uniformly around the T cell plasma membrane, creating a signaling platform inside and outside the immune synapse (Costello et al., 2002; Harriague and Bismuth, 2002). In the present study, fluorescent reporters have similarly been used to explore spatial and temporal aspects of DAG/PKD signaling in T cells. We show that PKD is cytosolic in unstimulated T cells, but that it rapidly polarizes to the immunological synapse when T cells respond to antigen/APC. DAG binding to PKD is essential for its membrane translocation. Moreover, the polarization of PKD to the T cell-APC contact zone is explained by the striking accumulation of DAG at the immunological synapse during T cell activation. There are, however, discrepancies between the sites of DAG accumulation and the localization of PKD, indicating that DAG levels are not the sole determinants of PKD localization, and that DAG accessibility to PKD is fine tuned by other regulatory mechanisms.

Results

PKD Transiently Translocates to the T Cell-APC Contact Zone

To examine PKD localization in T lymphocytes, we expressed an EGFP-tagged PKD construct (EGFP-PKD-1, Figure 1A) in Jurkat T cells and analyzed the intracellular localization of the enzyme in quiescent and TCR-activated cells by confocal microscopy, with both live-cell imaging and fixed samples. EGFP-tagged PKD responds normally to activation stimuli and colocalizes with endogenous PKD (Matthews et al., 2000a; Rey et al., 2001b). Figure 1B shows midsection confocal images of Jurkat cells expressing EGFP-PKD-1. In unstimulated cells, PKD is uniformly distributed throughout the cytosol and is excluded from the nucleus. Triggering of the TCR with CD3 antibodies induces a redistribution of PKD to the plasma membrane (Figure 1B). There was no obvious polarization of PKD, and the enzyme was distributed around the cell periphery.

To study the localization of PKD when the TCR is triggered by antigen/APCs, EGFP-PKD-1-expressing Jurkat cells were incubated with Raji B cells that were either unpulsed or pulsed with *Staphylococcus* enterotoxin E (SEE) superantigen. Figure 1C shows midsection confocal images of a T cell in a conjugate with an SEE-pulsed APC (bottom row) as well as T cells making random contacts with unpulsed APCs (top row). These data show that PKD repositions to the contact zone between the T cell and the antigen-pulsed APC. This was strictly antigen specific, as T cells mixed with nonpulsed APCs forming random contacts appear to be polarized toward the APC but do not recruit PKD to the contact zone (Figure 1C, top row).

Figure 2A shows a time course of the localization of PKD as T cells make contact with antigen/APC. The first contact between the T cell and the APC is frequently mediated by thin membrane protrusions (pseudopodia) of the APC. Strikingly, it can be seen that as the T cell makes this first contact with the pseudopodia of the APC, a rapid polarization of PKD toward the APC occurs (Figure 2A and Movie S1; see the Supplemental Data available with this article online). The T cell then undergoes a distinct morphological change, continues to polarize toward the APC, and then extends membrane onto the APC to form a cup-like structure that makes a tight contact zone between the two cells. As the APC contact continues, PKD continues to accumulate at the T cell-APC contact zone. The time course for PKD translocation to the APC contact zone was extremely rapid, with 64% of cells showing PKD accumulation in the contact area within 1 min of T cell-APC interaction.

Cell contacts that form between Jurkat T cells and SEE-pulsed Raji cells are sustained for several hours. However, repeated analysis of the kinetics of PKD translocation shows that this enzyme is only localized at the T cell-APC contact zone for around 10–15 min. A representative prolonged time course of the interaction between a T cell and the SEE-pulsed APC is shown in Figure 2B (also see Movie S2). During sustained contacts between the T cell and the APC, PKD relocalizes to the cytosol. Quantification of multiple experiments shows that translocation to the T cell-APC interaction zone is rapid, reaching a maximum within 1 min (Figure 2C); however, 15 min after the first contact, most of the protein has returned to the cytosol.

The mature immunological synapse is characterized by the segregation of receptors and signaling molecules into quite distinct concentric zones known as SMACs (Monks et al., 1998). The images in Figure 2D show

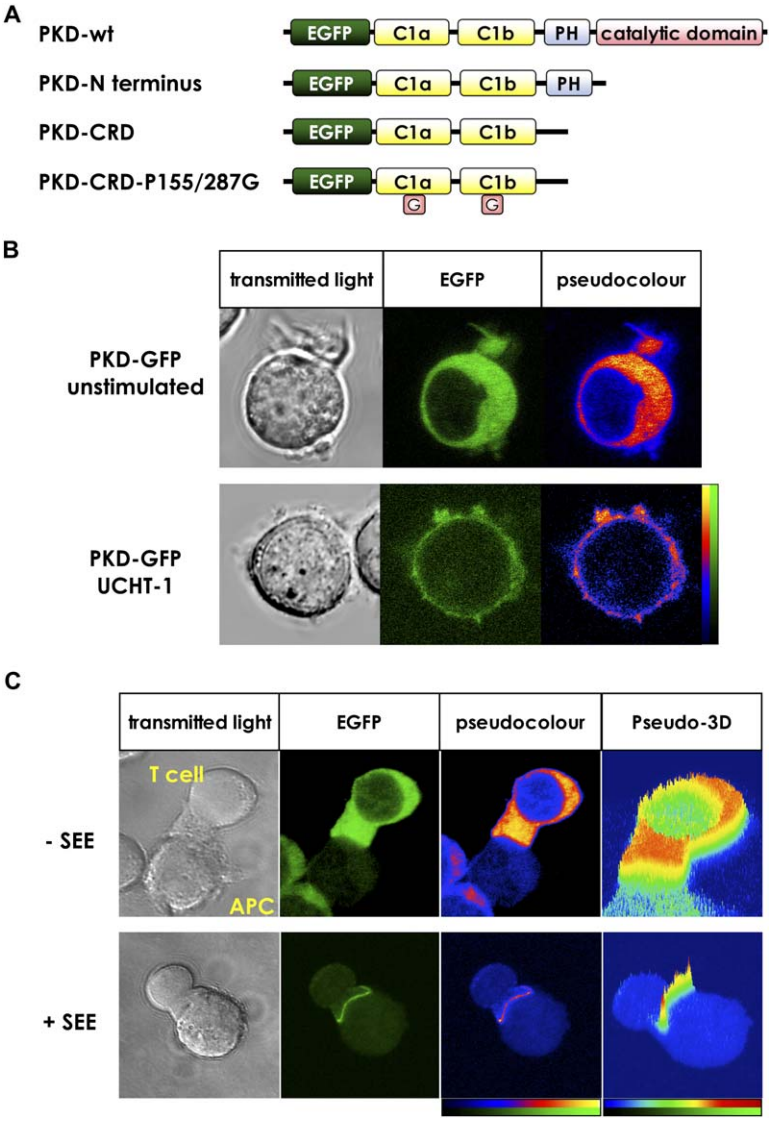

Figure 1. PKD Translocation upon T Cell Stimulation

(A) PKD constructs used.

(B) Jurkat T cells stably expressing wild-type EGFP-PKD were stimulated with TCR-cross-linking UCHT-1 antibody, and live images were taken before and 10 min after stimulation.

(C) Jurkat T cells expressing EGFP-PKD-1 (wt) were added to Raji TK-B cells either preloaded with SEE superantigen (bottom row) or unloaded as a control (top row). The fluorescence intensity is shown by pseudo colors as indicated in the calibration bar (third column) and a pseudo-colored pseudo 3D representation (fourth column). Images show a midsection of a representative cell.

three-dimensional reconstructions of PKD distribution in the T cell-APC contact zone and show that PKD does not segregate into any subarea. cSMAC formation matures 15–30 min after T cell activation (Lee et al., 2002). Accordingly, PKCθ, a classic cSMAC marker, is broadly distributed in the T cell-APC contact zone at a 10 min time point, whereas it accumulates in the c-SMAC at later time points (Figure 2E). Accordingly, cells have not formed defined cSMAC/pSMAC areas within the time course of PKD location to the synapse.

PKD Translocation to the T Cell-APC Contact Zone Is Mediated by the CRD

There are two tandem cysteine-rich motifs (C1a and C1b) within the CRD of PKD that are required for the enzyme to bind DAG or phorbol esters with high affinity. To explore the role of DAG binding to PKD for its translocation to the T cell-APC contact zone, we examined the localization of a PKD mutant containing substitutions of prolines 155 and 287 with glycine (GFP-PKD-P155/287G), which disables both the C1a and C1b domains of PKD, resulting in complete loss of DAG/phorbol ester binding (Iglesias et al., 1998). In these experiments, PKD localization was examined in T cells coexpressing DsRed2-tagged wild-type PKD (DsRed2-PKD-1) and EGFP-PKD-P155/287G, which allows for comparison of the localization of both constructs in the same cell. The data (Figure 3A) show that EGFP-PKD-P155/287G is cytosolic in unstimulated cells. To confirm that this mutant has lost DAG/phorbol ester binding, we examined the effect of phorbol-12,13-dibutyrate (PdBu) on the localization of EGFP-PKD-P155/287G. Figure 3A shows that PdBu induced plasma membrane translocation of DsRed2-tagged wild-type PKD-1, while the EGFP-tagged PKD-1-P155/287G mutant remained cytosolic. Figure 3B compares the localization of wild-type and PKD-P155/287G in T cells activated with SEE-pulsed APCs. Wild-type PKD relocalizes to the T cell-APC contact zone, whereas PKD-P155/287G remains cytosolic. Hence, the relocation of PKD to the T cell-APC contact zone is mediated by the CRD.

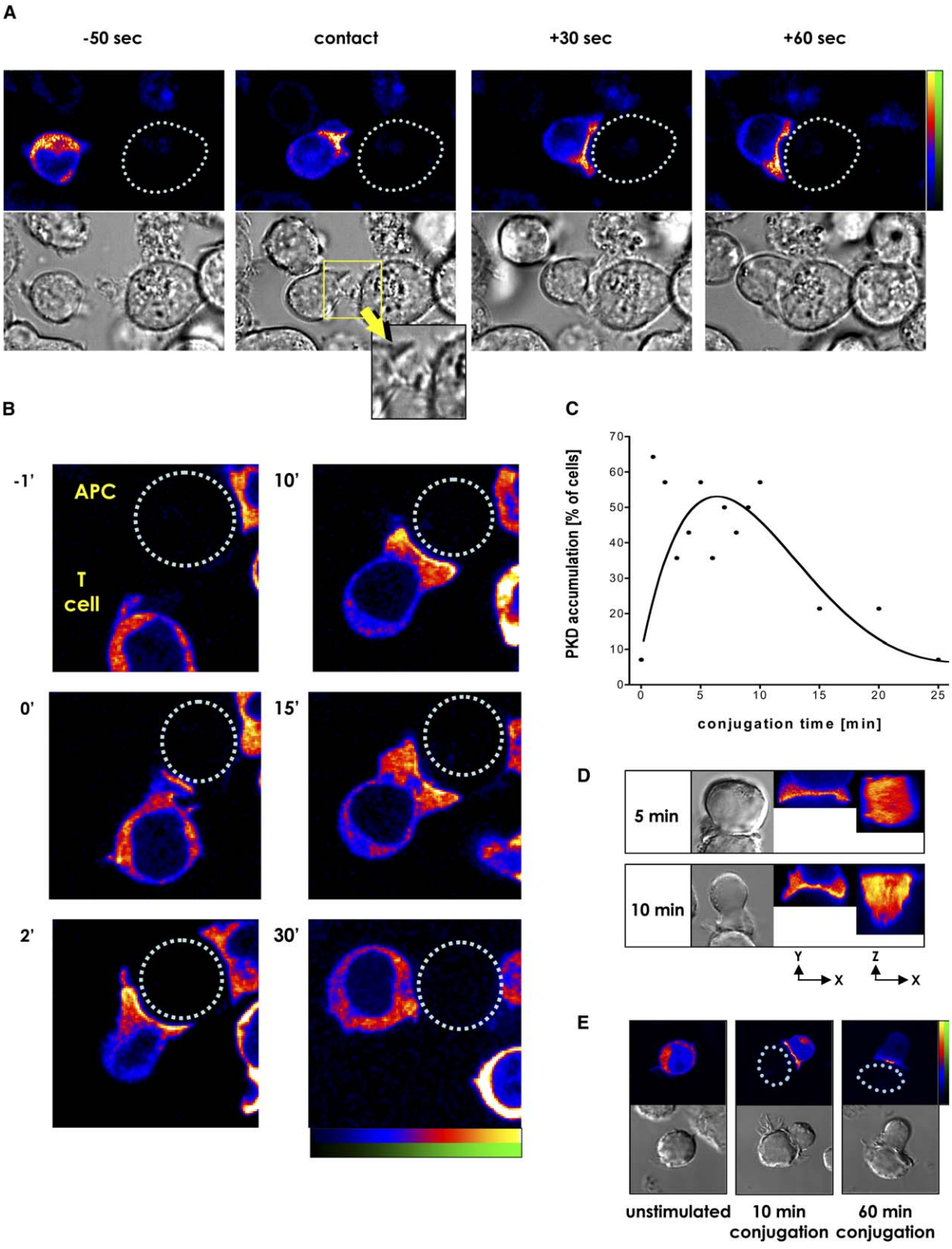

Figure 2. Kinetics and Distribution of PKD Translocated to the T Cell-APC Contact Zone

(A and B) Jurkat cells expressing wild-type EGFP-PKD-1 were added to superantigen-preloaded Raji B cells, and confocal images were taken at (A) 10 s or (B) 1 min intervals. Images show midsections of a representative time series. The full movies can be found in the Supplemental Data (Movies S1 and S2). (A) Polarization and recruitment of PKD to the T cell-APC interaction zone at first contact. The arrow in the transmitted-light image at the second time point indicates contact between the T cells and membrane extensions typical for the APC. The time interval between images was 10 s. (B) Long-term kinetics of transient translocation of PKD to the contact zone. Images were taken at 1 min intervals.

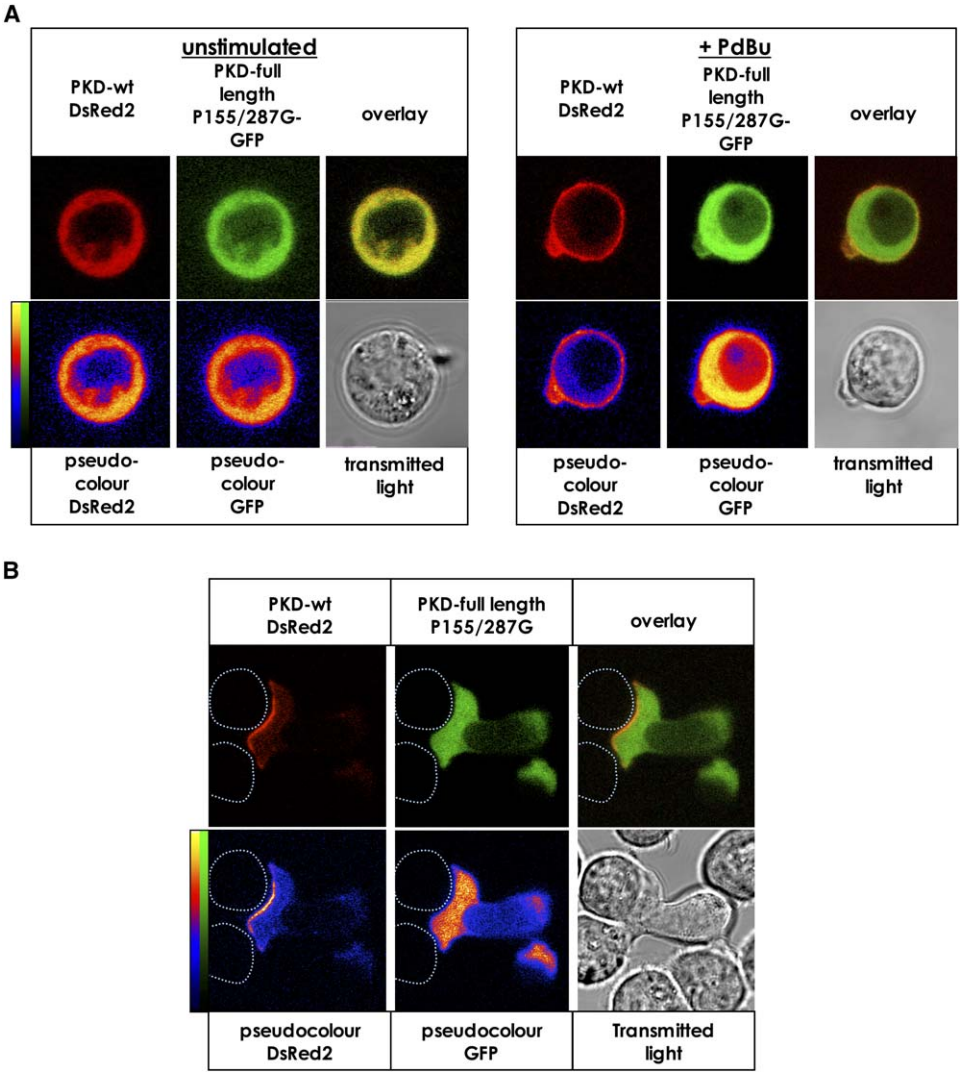

Figure 3. PKD Recruitment to the T Cell-APC Contact Zone Requires DAG Binding

(A and B) Jurkat T cells were transfected with wild-type DsRed2-PKD-1 and EGFP-PKD-P155/287G, and confocal images were taken of DsRed2 and EGFP fluorescence. The top-right images of each panel show the overlay of wild-type PKD and P155/287G. The bottom row shows false-color representations of DsRed2 (left) and EGFP (middle) and a transmitted-light image (right). (A) Effect of the CRD mutation P155/287G on the plasma membrane recruitment by PdBu. Cells were left untreated (left panel) or stimulated with 200 nM PdBu for 10 min (right panel). (B) Localization of wild-type DsRed2-PKD-1 and EGFP-PKD-1-P155/287G in T cells forming conjugates with superantigen-preloaded Raji B cells. The figure shows a representative image of a conjugate at maximal wild-type PKD translocation.

A Comparison of the Intracellular Localization of Wild-Type PKD and the Isolated PKD-CRD and N Terminus in Unstimulated Lymphocytes

The requirement of an intact DAG binding a CRD for recruitment of PKD to the immunological synapse raised the question of whether DAG binding alone is sufficient to reposition PKD when T cells make contact with APCs. Previous studies have used EGFP-tagged CRDs as fluorescent indicators to monitor intracellular DAG in living cells (Carrasco and Merida, 2004; Oancea et al., 1998), but DAG localization under physiological T cell stimulation with APCs has never been investigated. Accordingly, we examined the localization of an EGFP-tagged fusion protein of the N-terminal PKD-CRD (amino acid residues 1–365) to determine the distribution of intracellular DAG in quiescent or activated T cells.

(C) Quantification of PKD recruitment to the T cell-APC interaction zone. A total of 248 single images from 15 movies generated in 9 independent experiments were analyzed. Values show the percentage of cells with an accumulation of EGFP in the contact area higher than 1.15 relative to a neighboring cytoplasmic area.

(D) Distribution of PKD within the synapse. 3D reconstructions were generated from z-stack confocal images of T cell-APC synapses fixed at the indicated time points. The image shows a DIC image (left), a side view (middle), and an en-face view (right) of representative cells at two different time points.

(E) Localization of PKCθ (wt) in resting and stimulated T cells. EGFP-labeled PKCθ was expressed in Jurkat cells, and images were taken in unstimulated cells and after conjugation with antigen-loaded Raji B cells as described above. Representative images at different time points of conjugation are shown.

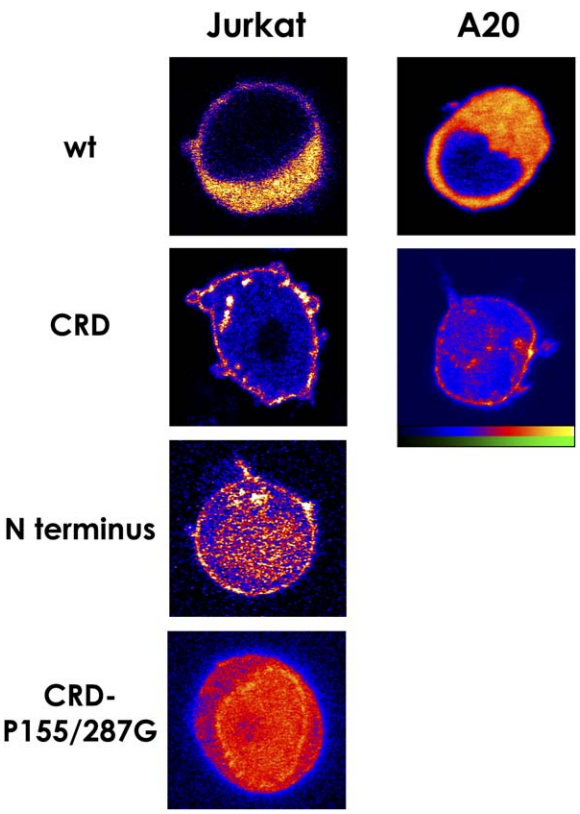

Figure 4. Comparison of the Localization of Wild-Type PKD with the Isolated N Terminus and the CRD

Different constructs of PKD fused to EGFP were expressed in Jurkat T cells (left) and A20 B cells (right), and localization of the proteins was observed in unstimulated cells using confocal microscopy. Images show false-color representations of EGFP fluorescence and are representative for at least 20 images of each construct.

Strikingly, the PKD-CRD was constitutively localized to the plasma membrane in unstimulated T cells (Figure 4). This pattern of localization required DAG binding, as the CRD-P155/287G mutant is not at the plasma membrane but diffusely distributed throughout the cytosol and the nucleus (Figure 4, bottom row). The plasma membrane recruitment of the CRD thus needs an intact DAG binding domain. Constitutive association of an isolated PKD-CRD with the plasma membrane has not been reported in fibroblasts and HeLa cells: in the latter, the CRD associated with the trans-Golgi network (Maeda et al., 2001). However, constitutive plasma membrane association of the PKD-CRD was not restricted to the T cells used herein, but was also seen in B lymphocytes. Accordingly, the data in Figure 4 compare the intracellular localization of EGFP-tagged wild-type PKD and the isolated EGFP-PKD-CRD in Jurkat cells and in the A20 B lymphoma cell line: wild-type PKD is cytosolic, but the isolated PKD-CRD is constitutively at the plasma membrane in both T and B cells.

The Intracellular Distribution of the EGFP-Tagged PKD-CRD in Antigen-Activated T Cells

EGFP-tagged CRDs have been used to monitor real-time changes in DAG production in single cells (Oancea et al., 1998). Accordingly, we examined whether there was any change in the intracellular position of an EGFP-tagged PKD-CRD in T cells activated by antigen/APC. The experiments depicted in Figure 5A show images of a T cell expressing the EGFP-tagged PKD-CRD making contacts with an SEE-pulsed APC. These data show that there is a rapid accumulation of the PKD-CRD into the contact area between the T cells and the APC. This is a rapid response, and within an average of 2 min, the EGFP-tagged PKD-CRD is no longer generally distributed around the cell periphery, but rather is focused in the T cell-APC contact zone. The redistribution of the PKD-CRD into the contact zone is a prolonged response, unlike the transient redistribution of full-length PKD. Hence, even 60–100 min after the T cell contact with the APC is formed, the PKD-CRD is still concentrated in the contact area of the cells, whereas full-length wild-type PKD relocalizes to the cytosol during sustained T cell activation. One other observation concerning the distribution of the PKD-CRD after prolonged antigen receptor stimulation is that in addition to its focus at the T cell-APC contact zone, it also appears in vesicular structures that seem to bud off from the contact area and move into the cell interior (see Figure 5A and Movie S3). Full-length PKD is never seen in such structures, nor is the PKD-1 CRD-P155/287G mutant that has lost the ability to bind DAG.

One other characteristic feature of CRD localization at the immune synapse is that it appears to focus centrally rather than showing the diffuse distribution typically seen with full-length PKD. The images in Figure 5B show a three-dimensional reconstruction of the T cell-APC contact zone formed between T cells expressing an EGFP-tagged PKD-CRD and antigen-pulsed APC, at 50 min from conjugate formation. These images show that the CRD is clearly focused in the center of the synapse, the cSMAC, and is excluded from the outer area.

The change in distribution of EGFP-tagged PKD-CRD when T cells are activated by antigen/APC indicates that, during T cell activation, a gradient of DAG forms with a high level of DAG accumulation in the cSMAC. To explore this hypothesis further, we repeated these experiments with an EGFP-labeled CRD probe of PKCθ. This probe is constitutively localized and uniformly distributed at the plasma membrane of unstimulated T cells (Figure 6, top row). In contrast, wild-type PKCθ is mostly cytosolic (Figure 2E). Figure 6 also shows images of T cells expressing EGFP-tagged PKCθ-CRD making contacts with SEE-pulsed APC. These data show that within 2 min, EGFP-tagged PKCθ-CRD is no longer generally distributed around the cell periphery, but rather is focused in the T cell-APC contact zone. This recruitment to the synapse was prolonged and associated with accumulation in the central synapse at later time points. The experiments with the PKCθ-CRD thus support the model that a gradient of DAG is formed during T cell activation, and that the immune synapse is a site of DAG accumulation.

The Role of PKD Activity in Regulating Its Intracellular Localization

The failure of full-length PKD and the PKD-CRD to colocalize in both unstimulated and activated T cells indicates that DAG is not the sole signal to determine the intracellular position of this enzyme. To further probe the

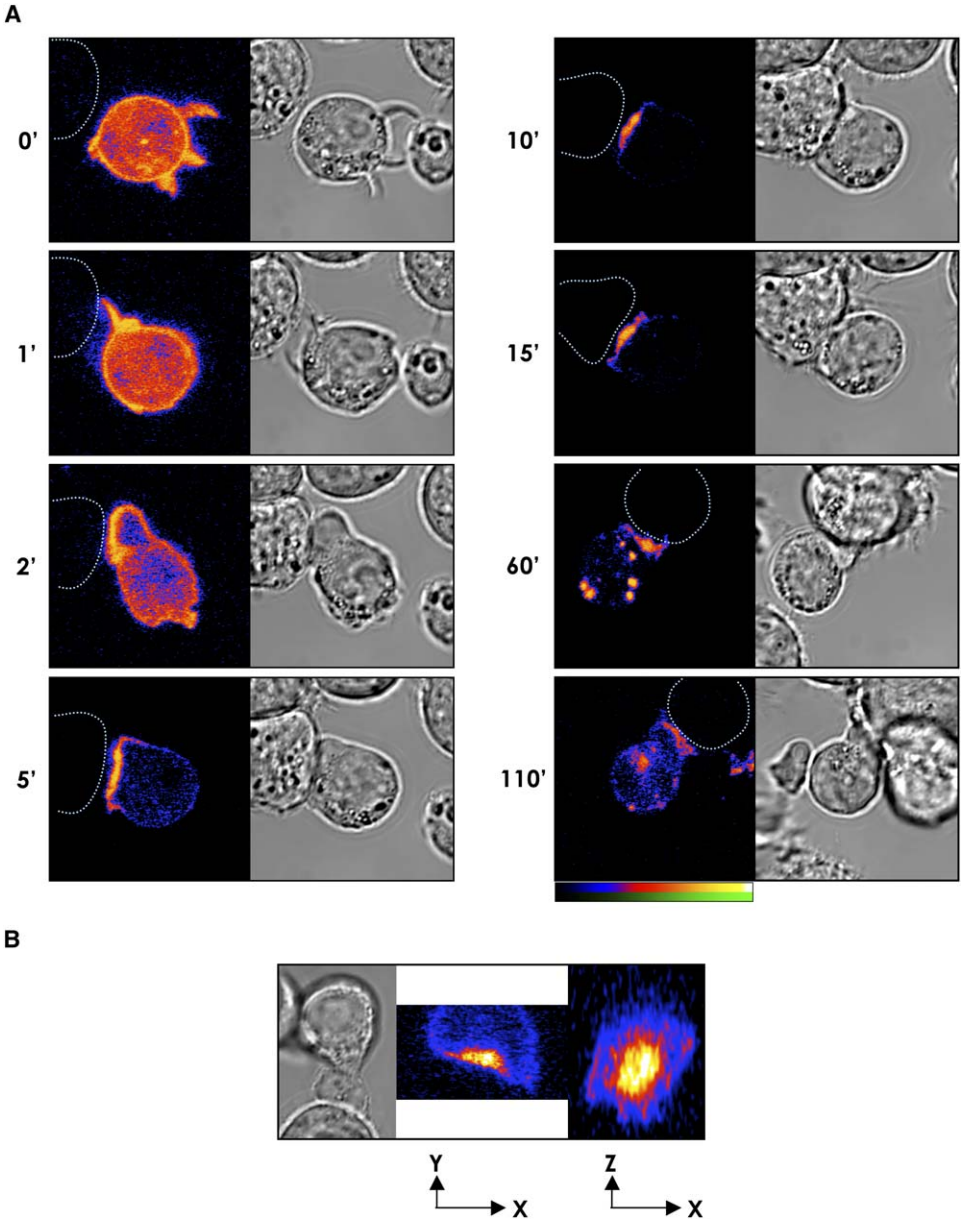

Figure 5. Localization of EGFP-Tagged PKD-CRD in T Cells Stimulated by APCs

An EGFP fusion construct of the isolated CRD of PKD was transiently transfected into Jurkat T cells, the cells were sorted and allowed to form conjugates with superantigen-preloaded Raji B cells, and live confocal images were taken at 1 min intervals during conjugate formation. Images show a midsection of single images representative of 3 time series and 30 single images taken at different time points of conjugation.

(A) PKD-CRD localization during T cell-APC conjugation.

(B) 3D reconstruction of the PKD localization in the T cell-APC interaction zone 50 min after conjugate formation. Z-stacks of conjugates were used for 3D reconstruction with Zeiss LSM software. Images show a transmitted-light image (left) as well as a midsection image (middle) and an en-face view of the 3D reconstruction (right) in false colors. The full movie is shown in Movie S3.

mechanisms that control PKD localization in T cells, we examined the intracellular distribution of a construct comprising the entire N-terminal regulatory domain of PKD (amino acid residues 1–558) that includes a PH domain but lacks the kinase catalytic domain. The PKD N-terminal probe showed a similar localization profile as the PKD-CRD in both quiescent and antigen receptor-activated cells: constitutive plasma membrane association in unstimulated lymphocytes, a redistribution into the center of the T cell-APC contact zone for a prolonged period of time, and an appearance in vesicular structures in the cell interior during the sustained response to antigen/APC (Figure 7A and Movie S4). These data indicate that the PKD catalytic domain plays a role in regulating the localization of the enzyme. Accordingly, we examined the localization of a catalytically inactive PKD construct with a mutation in its ATP binding site. Figure 7B shows that this kinase-dead PKD mutant can be found in the cytosol of unstimulated T cells, but it also shows partial accumulation at the plasma membrane and in a perinuclear vesicular structure. This is very different to the distribution of the wild-type enzyme.

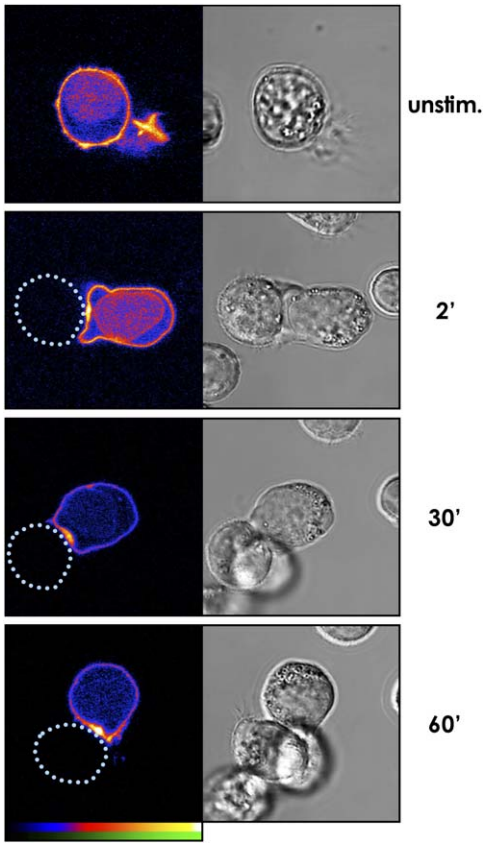

Figure 6. Subcellular Localization of the PKCθ-CRD

Jurkat T cells transiently transfected with EGFP-tagged PKCθ-CRD were stimulated with superantigen-loaded Raji B cells. Confocal live cell images were taken during T cell-APC conjugation, and representative still images of unstimulated as well as APC-stimulated cells are shown.

When T cells make contacts with antigen-primed APCs, the kinase-dead PKD becomes concentrated in the T cell-APC contact zone where it resides for a prolonged time. It appears to focus at the center of the synapse at the cSMAC, which is similar to the distribution of the isolated PKD-CRD and quite distinct from the localization pattern of the full-length enzyme (Figure 7B and Movie S5).

Discussion

Lipid metabolites act as key second messengers for T cells, and, hence, there is considerable interest in their subcellular localization during immune stimulation. Herein, we have used probes comprising the EGFP-tagged CRD of PKD or PKCθ to visualize DAG localization as T cells make contacts with APCs. These DAG probes constitutively localize to the plasma membrane in unstimulated lymphocytes, but they relocate to the contact zone between the T cell and the antigen-primed APC. Hence, the immune synapse strikingly acts as a site for DAG accumulation. T cells make sustained contacts with APC, and during this time the DAG probes focus to the central synapse core. The presence of the PKD and PKCθ-CRD probes at the plasma membrane of unstimulated T cells reveals the existence of basal pools of intracellular DAG in T cell membranes. The presence of high basal levels of DAG in quiescent cells has been described previously (Jones et al., 1999; Pettitt et al., 1997). Indeed, it has always been a puzzle as to how relatively small mass changes in DAG that are produced by PI-(4,5)-P_2 hydrolysis are able to act as a signaling switch, given this background of high basal DAG. The present data shed some insight by revealing that a DAG gradient is formed after T cell activation. In quiescent cells, DAG is uniformly distributed around the plasma membrane, whereas after T cell activation with antigen/APC the cSMAC strikingly acts as a site for DAG accumulation. The spatial concentration of DAG to a focused area of the T cell membrane is a mechanism that will establish a sharp gradient of DAG levels within an activated T cell without substantial changes in total DAG mass.

The accumulation of DAG in the immune synapse is in marked contrast to the membrane distribution of another key lipid second messenger, PtdIns-(3,4,5)-P_3. It has been shown that after T cell triggering with antigen/APC, PtdIns-(3,4,5)-P_3 localizes relatively uniformly around the T cell plasma membrane, creating a signaling platform inside and outside the immune synapse (Costello et al., 2002; Harriague and Bismuth, 2002). Hence, DAG and PtdIns-(3,4,5)-P_3 signaling occur in spatially distinct areas of the T cell plasma membrane. The rationale for exploring DAG localization in T cells is that understanding DAG spatial distribution is fundamental for understanding the mechanisms that control the subcellular distribution of DAG effectors. In this context, we show that the DAG binding protein PKD relocates to the plasma membrane and transiently focuses at the T cell-APC contact zone in response to antigen/APC stimulation. This repositioning of PKD requires an intact DAG binding domain. However, if localized increases in DAG were the sole explanation for PKD translocation, then the residence of wild-type PKD at the plasma membrane or at the T cell-APC contact zone would coincide with the residence of the isolated PKD-CRD. This does not happen: in quiescent T cells, the CRD probe constitutively localizes to the plasma membrane, whereas wild-type PKD is cytosolic. Moreover, during sustained T cell activation, the CRD accumulates and persists in the cSMAC, whereas PKD returns to the cytosol within 15 min of conjugate formation. These discrepancies must reflect that DAG accessibility to the CRD within full-length PKD is regulated either by conformational changes in the enzyme or by PKD binding to other cellular proteins. For example, there must be sufficient DAG at the plasma membrane of unstimulated lymphocytes to recruit PKD-CRD, but the retention of wild-type PKD in the cytosol indicates that the CRD is "masked" in the inactive enzyme, thereby preventing DAG binding and membrane translocation. Hence, the movement of PKD to the plasma membrane appears to involve some conformational change that unmasks the CRD, allowing DAG binding; PKD polarization to the synapse would also be explained by localized increases in DAG concentration at this area. The model that the DAG binding sites in PKD are not freely accessible in quiescent cells also applies to PKCθ. Wild-type PKCθ is cytosolic in quiescent T cells, whereas the isolated PKCθ-CRD

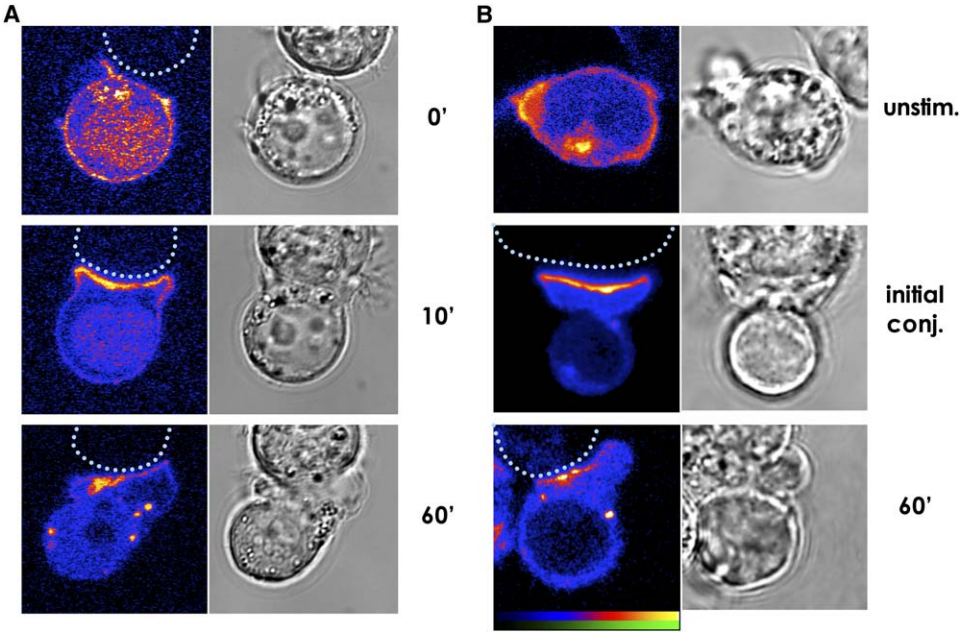

Figure 7. Effect of the Catalytic Activity on the Localization of PKD

(A) Localization of EGFP-PKD-N-terminus in Jurkat T cells at the indicated time points of conjugation with superantigen-preloaded Raji B cells. Images shown are representative for at least 3 time series and 20 single images at different time points of conjugation. The full movie is shown in Movie S4.

(B) Localization of kinase-dead EGFP-PKD-D733A in unstimulated Jurkat T cells and upon conjugation with superantigen-preloaded Raji B cells. Images are representative for three independent movies. The full movie is shown in Movie S5.

constitutively localizes to the membrane. Hence, DAG availability to the PKCθ-CRD must be masked in quiescent cells.

PKD returns to the cytosol within 15 min of T cell-APC conjugate formation, whereas the translocation of the CRD to the contact zone is sustained. Moreover, while the CRD concentrates in the central zone during synapse maturation, the full-length enzyme is diffusely spread throughout the contact area and returns to the cytosol before the cSMAC accumulation of the CRD becomes evident. Accordingly, there must be factors that limit the accessibility of PKD to DAG in the immune synapse. One option we explored was whether PKD catalytic activity plays a role in regulating PKD localization. We therefore studied the intracellular distribution of kinase-dead PKD-D733A. When T cells make contacts with an antigen-primed APC, PKD-1-D733A translocated to the immune synapse and, unlike the wild-type enzyme, accumulated at the contact zone for a sustained time. PKD activity thus plays a role in regulating the enzyme's intracellular localization. This could reflect that PKD autophosphorylation causes conformational changes altering the accessibility of the CRD, causing release from DAG docking or phosphorylation of another interaction partner.

Studies of the spatial distribution of PKD not only establish some novel concepts about DAG signal transduction, but they are also relevant to understanding how a serine kinase can have multiple functions within a cell. Thus, although PKD membrane translocation is transient, PKD activation is sustained (Matthews et al., 2000a; Wood et al., 2005). The sequence of PKD movements thus allows this enzyme to contact different substrates at different phases of T cell activation and hence have temporally distinct functions. A striking feature of PKD translocation to the T cell-APC contact zone is its speed. The rapidity with which PKD polarizes into the contact zone thus precedes the time course for the formation of a fully differentiated mature synapse (Ehrlich et al., 2002; Lee et al., 2002; Qi et al., 2001). Recent data have shown that PKD-1 controls the activity and plasma membrane localization of the GTPase Rap1, which regulates the activity of integrins (Medeiros et al., 2005). Integrin activation is an essential part of the signaling processes that switch T cells from a mobile antigen-scanning state to a cell that forms a stable conjugate with an APC. Accordingly, the ability of PKD to regulate integrins (Medeiros et al., 2005) and the kinetics of PKD polarization would be consistent with PKD having a role in organizing a signaling platform to form the synapse. What are PKD substrates at the immune synapse? The Ste20-related kinase HPK1, which is known to accumulate at the immune synapse, can be phosphorylated and activated by PKD-1 (Arnold et al., 2005). PKD-1 translocation to the immune synapse could thus allow it to regulate this kinase. What are cytosolic PKD substrates? One example is HDAC7, which interacts with MEF2 transcription factors, resulting in repression of their transcriptional activity (Dequiedt et al., 2005; Matthews et al., 2006; Parra et al., 2005). The repressive activity of HDACs is relieved after PKD-mediated phosphorylation, which promotes nuclear export of these proteins. HDAC7 shuttles between the nucleus and the cytosol and has never been seen at the plasma membrane. Hence, PKD-1 translocation to the immune synapse could delay HDAC phosphorylation

until the T cell enters a sustained phase of T cell activation and stimulated PKD-1 relocates to the cytosol. The movement of PKD between the immune synapse and the T cell cytosol could thus be a device that temporally restricts PKD access to different substrates and allows membrane-targeted and cytosolic PKD-1 to have different functions at different phases of T cell activation. Whether there is similar spatial/temporal regulation of PKD3 activity in the context of the synapse will be interesting to determine.

In summary, the present study provides a paradigm for TCR induction of DAG signal transduction that explains how small changes in DAG total mass can function as a signaling switch. Unstimulated T cells have high basal levels of DAG, but these are uniformly distributed around the plasma membrane. After T cell activation, a gradient of DAG is created with a focused concentration of this lipid at the immunological synapse. Moreover, DAG binding sites in DAG effectors are not freely accessible, and modifications by other factors are required to allow their recruitment to DAG-rich foci in the synapse. DAG is thus not a simple on/off signal switch, but it is fine-tuned by spatial and temporal concentration changes and structural modifications of its effectors. The present conclusions are based on the use of PKD-1 and PKCθ as models for DAG signal transduction, and it should be noted that the CRDs in different proteins have different selectivities for various molecular species of DAG (Madani et al., 2001). Accordingly, experiments that compare the localization patterns of different CRDs may be able to visualize additional foci of DAG accumulation in antigen-activated T cells. Nevertheless, the concentration of DAG in the cSMAC revealed herein argues that the immunological synapse has a unique signaling function in T cells in the regulation of DAG signal transduction.

Experimental Procedures

Cell Lines, Plasmids, and Transfections

Jurkat T cells, Raji B cells, and A20 B cells were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum. EGFP-labeled constructs of mouse PKD-1 were described previously (Matthews et al., 1999; Wood et al., 2005). EGFP-PKD-1-CRD (amino acids 1–365), EGFP-PKD-1-CRD-P155/287G (PKD-CRD with one single point mutation in each C1 domain, abolishing DAG and phorbol ester binding [Iglesias et al., 1998]), EGFP-PKD-1-N-terminus (amino acids 1–558), and kinase-dead EGFP-PKD-1-D733A were constructed by subcloning the respective PKD constructs into the vector pEF-EGFP (Matthews et al., 1999). DsRed2-PKD-1 was generated by subcloning PKD-1 into the vector pDsRed2 (Clontech).

The wild-type construct of PKCθ-EGFP was kindly provided by Gottfried Baier, Innsbruck Medical University, Austria. For the generation of the EGFP-labeled version of PKCθ-CRD, the CRD of PKCθ was amplified by PCR with primers containing an EcoRI and SacII restriction site in the 5′ and 3′ primers, respectively. Using these restriction sites, the PKCθ-CRD was inserted into a modified version of the pEGFP-C1 vector (Clontech) containing an additional polylinker sequence.

For transfections, 10 million cells were centrifuged at 150 × g for 5 min, resuspended in RPMI 1640 medium without FCS, and transferred to 4 mm electroporation cuvettes (BioRad Gene Pulser Xcell), and 15 μg plasmid DNA was added. Electroporation was carried out with a BioRad Gene Pulser at 0.31 V/960 μF. Cells were then transferred to 16 ml fresh, prewarmed RPMI 1640 medium containing 10 % FCS and incubated overnight. For the generation of a Jurkat cell line stably expressing EGFP-PKD-1, Jurkat cells were cotransfected with pcDNA3, and transfected cells were sorted on a fluorescence-activated cell sorter for EGFP expression. A stable cell line was then maintained by cultivation in the presence of 2 μg/ml geneticin (Calbiochem). Cells were sorted for intermediate EGFP expression, quantified to be in the range of 10^5–10^6 molecules per cell by using a flow cytometer and standard beads coated with known numbers of GFP molecules. This expression level corresponds to the level of endogenous PKD expression, thus avoiding artifacts induced by high overexpression of the transfected protein.

Cell Stimulation and Conjugation

Cells were seeded onto glass coverslips in 24-well plates in conjugation medium (RPMI medium without phenol red/0.5 % FCS) and allowed to attach for 20 min at 37ºC. T cells were treated with either 200 nM phorbol-12,13-dibutyrate (PdBu, Calbiochem) or 10 μg/ml of the CD3 antibody UCHT-1. For conjugate formation, Raji B cells at a density of 5 × 10^6/ml were preloaded with *Staphylococcus* enterotoxin E (SEE, Toxin Technology) at a concentration of 10 ng/ml in full growth medium for 1 hr. Cells were washed 3 times with RPMI, resuspended in conjugation medium at 2.5 × 10^6/ml, and seeded onto glass coverslips. A20 B cells were grown overnight on glass coverslips (diameter 13 mm) at a density of 500,000 cells per coverslip. For fixation, cells were seeded onto glass coverslips in 24-well plates, while, for live cell imaging, cells were seeded into MatTek glass-bottom dishes (MatTek). Cells were allowed to attach for 20 min at 37ºC, washed 3 times with medium, and overlaid with 1 or 2 ml conjugation medium (coverslips or MatTek dishes, respectively). Conjugation was started by addition of 500,000 T cells, and the cells were incubated at 37ºC.

Cell Fixation

Fixing agent was freshly prepared by dissolving 4% paraformaldehyde plus 2% sucrose in phosphate-buffered saline (PBS), heating to 65ºC for 10 min, adding 1/60 volume 1 M NaOH, and incubating further at 65ºC for 10 min. The pH was readjusted at room temperature to pH 7.4 with 1 M HCl. At the end of the indicated incubation time, the medium was replaced by fixing agent, and the cells were incubated at room temperature for 5 min. Fixation was stopped by washing once with PBS plus 0.1 M ammoniumchloride and three times with PBS. Coverslips were rinsed in distilled H_2O and quickly air dried. Then, 10 μl ProLong Antifade (Molecular Probes) was added to microscopy slides, and the coverslips were mounted. The mounting medium was allowed to dry at room temperature in the dark overnight. Subsequently, the slides were stored at 4ºC.

Fluorescence-Activated Cell Sorting

Transiently transfected cells were incubated overnight, centrifuged at 150 × g for 5 min, and resuspended in 2 ml complete medium. EGFP-expressing cells were sorted on a FACS Vantage SE (Beckton Dickinson), recovered in complete RPMI 1640 medium, and incubated overnight in RPMI/10% FCS before live imaging.

Confocal Microscopy

Images were acquired on a Zeiss LSM-510 upright or inverted microscope for fixed cell or live imaging, respectively. All images were taken with a 63× Plan Apochromat Ph3 objective lens. Excitation/emission settings for different fluorescent labels were as follows: EGFP, 488 nm/BP 505–530 nm; DsRed2, 543 nm/LP 585 nm. For three-dimensional reconstructions, z-stacks were taken at intervals of 0.3–0.7 μm. Using the Zeiss LSM software, a panoramic three-dimensional projection was generated. For live imaging, cells were seeded into MatTek glass-bottom dishes at a density of 0.5 ×10^6 per dish in conjugation medium and incubated at 37ºC for 20 min. The medium was then replaced by 2 ml conjugation medium, and the cells were incubated at 37ºC/5% CO_2 on an inverted microscope. Images were taken at the time intervals indicated in the figure legends. Cells were stimulated by the addition of 1 ml 3× concentrated PdBu or UCHT-1 in conjugation medium. For live imaging of conjugate formation, Raji B cells were preloaded with superantigen as described above, washed three times with conjugation medium, and seeded into MatTek dishes at 250,000 cells in 200 μl medium per dish. After incubation at 37ºC for 20 min, the medium was replaced by 2 ml fresh medium, and the dish was incubated at 37ºC/5% CO_2 on an inverted microscope for the duration of the experiment. Jurkat cells expressing fluorescence-labeled PKD constructs were added

at 500,000 cells per dish, and images were taken at the time intervals indicated in the figure legends.

Image Analysis

Image analysis was performed in Adobe Photoshop CS. Background was subtracted by using false-color gradient maps and levels in adjustment layers. Cellular structures were selected semiautomatically by using threshold adjustment layers in combination with the lasso tool. Image quantification was performed on the underlying original, unmodified images. For quantification of PKD translocation to the T cell contact area, the ratio of mean intensity of the contact area versus an equivalent area in the underlying cytosol was calculated, and a ratio >1.15 was regarded as EGFP accumulation. Visibility of structures in false-color images for presentation was improved by application of a 0.3 pixel Gaussian blur. Visibility of differential interference contrast (DIC) images was improved by using gradient blur, unsharp masks, levels, and curves. For videos, images were adjusted in Photoshop by using cropping, background subtraction, and false colors and were converted to Quicktime movies by using GraphicConverter X 5.5 (Lemkesoft, http://lemkesoft.com) with MPEG-4 compression in medium quality, 32 bit color depth, and the pixel dimensions of the original files.

Supplemental Data

Supplemental Data include the full movies for Figures 2A, 2B, 5A, 7A, and 7B (Movies S1–S5, respectively) and are available with this article online at http://www.immunity.com/cgi/content/full/24/5/535/DC1/.

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References

Arnold, R., Patzak, I.M., Neuhaus, B., Vancauwenbergh, S., Veillette, A., Van Lint, J., and Kiefer, F. (2005). Activation of hematopoietic progenitor kinase 1 involves relocation, autophosphorylation, and transphosphorylation by protein kinase D1. Mol. Cell. Biol. *25*, 2364–2383.

Bivona, T.G., Perez De Castro, I., Ahearn, I.M., Grana, T.M., Chiu, V.K., Lockyer, P.J., Cullen, P.J., Pellicer, A., Cox, A.D., and Philips, M.R. (2003). Phospholipase Cγ activates Ras on the Golgi apparatus by means of RasGRP1. Nature *424*, 694–698.

Bromley, S.K., Burack, W.R., Johnson, K.G., Somersalo, K., Sims, T.N., Sumen, C., Davis, M.M., Shaw, A.S., Allen, P.M., and Dustin, M.L. (2001). The immunological synapse. Annu. Rev. Immunol. *19*, 375–396.

Carrasco, S., and Merida, I. (2004). Diacylglycerol-dependent binding recruits PKCθ and RasGRP1 C1 domains to specific subcellular localizations in living T lymphocytes. Mol. Biol. Cell *15*, 2932–2942.

Costello, P.S., Gallagher, M., and Cantrell, D.A. (2002). Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse. Nat. Immunol. *3*, 1082–1089.

Davis, D.M., and Dustin, M.L. (2004). What is the importance of the immunological synapse? Trends Immunol. *25*, 323–327.

Dequiedt, F., Van Lint, J., Lecomte, E., Van Duppen, V., Seufferlein, T., Vandenheede, J.R., Wattiez, R., and Kettmann, R. (2005). Phosphorylation of histone deacetylase 7 by protein kinase D mediates T cell receptor-induced Nur77 expression and apoptosis. J. Exp. Med. *201*, 793–804.

Diaz-Flores, E., Siliceo, M., Martinez, A.C., and Merida, I. (2003). Membrane translocation of protein kinase Cθ during T lymphocyte activation requires phospholipase C-γ-generated diacylglycerol. J. Biol. Chem. *278*, 29208–29215.

Ebinu, J.O., Stang, S.L., Teixeira, C., Bottorff, D.A., Hooton, J., Blumberg, P.M., Barry, M., Bleakley, R.C., Ostergaard, H.L., and Stone, J.C. (2000). RasGRP links T-cell receptor signaling to Ras. Blood *95*, 3199–3203.

Ehrlich, L., Ebert, P., Krummel, M., Weiss, A., and Davis, M. (2002). Dynamics of p56lck translocation to the T cell immunological synapse following agonist and antagonist stimulation. Immunity *17*, 809–822.

Friedl, P., den Boer, A.T., and Gunzer, M. (2005). Tuning immune responses: diversity and adaptation of the immunological synapse. Nat. Rev. Immunol. *5*, 532–545.

Grakoui, A., Bromley, S.K., Sumen, C., Davis, M.M., Shaw, A.S., Allen, P.M., and Dustin, M.L. (1999). The immunological synapse: a molecular machine controlling T cell activation. Science *285*, 221–227.

Harriague, J., and Bismuth, G. (2002). Imaging antigen-induced PI3K activation in T cells. Nat. Immunol. *3*, 1090–1096.

Iglesias, T., Matthews, S., and Rozengurt, E. (1998). Dissimilar phorbol ester binding properties of the individual cysteine-rich motifs of protein kinase D. FEBS Lett. *437*, 19–23.

Jones, D.R., Pettitt, T.R., Sanjuan, M.A., Merida, I., and Wakelam, M.J. (1999). Interleukin-2 causes an increase in saturated/monounsaturated phosphatidic acid derived from 1,2-diacylglycerol and 1-O-alkyl-2-acylglycerol. J. Biol. Chem. *274*, 16846–16852.

Kane, L.P., Lin, J., and Weiss, A. (2000). Signal transduction by the TCR for antigen. Curr. Opin. Immunol. *12*, 242–249.

Kazanietz, M.G. (2002). Novel "nonkinase" phorbol ester receptors: the C1 domain connection. Mol. Pharmacol. *61*, 759–767.

Kupfer, A., and Kupfer, H. (2003). Imaging immune cell interactions and functions: SMACs and the immunological synapse. Semin. Immunol. *15*, 295–300.

Lee, K.H., Holdorf, A.D., Dustin, M.L., Chan, A.C., Allen, P.M., and Shaw, A.S. (2002). T cell receptor signaling precedes immunological synapse formation. Science *295*, 1539–1542.

Madani, S., Hichami, A., Legrand, A., Belleville, J., and Khan, N.A. (2001). Implication of acyl chain of diacylglycerols in activation of different isoforms of protein kinase C. FASEB J. *15*, 2595–2601.

Maeda, Y., Beznoussenko, G.V., Van Lint, J., Mironov, A.A., and Malhotra, V. (2001). Recruitment of protein kinase D to the trans-Golgi network via the first cysteine-rich domain. EMBO J. *20*, 5982–5990.

Marklund, U., Lightfoot, K., and Cantrell, D. (2003). Intracellular location and cell context-dependent function of protein kinase D. Immunity *19*, 491–501.

Matthews, S., Iglesias, T., Cantrell, D., and Rozengurt, E. (1999). Dynamic re-distribution of protein kinase D (PKD) as revealed by a GFP-PKD fusion protein: dissociation from PKD activation. FEBS Lett. *457*, 515–521.

Matthews, S.A., Iglesias, T., Rozengurt, E., and Cantrell, D. (2000a). Spatial and temporal regulation of protein kinase D (PKD). EMBO J. *19*, 2935–2945.

Matthews, S.A., Rozengurt, E., and Cantrell, D. (2000b). Protein kinase D. A selective target for antigen receptors and a downstream target for protein kinase C in lymphocytes. J. Exp. Med. *191*, 2075–2082.

Matthews, S.A., Liu, P., Spitaler, M., Olson, E.N., McKinsey, T.A., Cantrell, D.A., and Scharenberg, A.M. (2006). Essential role for protein kinase D family kinases in the regulation of class II histone deacetylases in B lymphocytes. Mol. Cell. Biol. *26*, 1569–1577.

Medeiros, R.B., Dickey, D.M., Chung, H., Quale, A.C., Nagarajan, L.R., Billadeau, D.D., and Shimizu, Y. (2005). Protein kinase D1 and the β1 integrin cytoplasmic domain control β1 integrin function via regulation of Rap1 activation. Immunity *23*, 213–226.

Monks, C.R., Kupfer, H., Tamir, I., Barlow, A., and Kupfer, A. (1997). Selective modulation of protein kinase C-theta during T-cell activation. Nature *385*, 83–86.

Monks, C.R., Freiberg, B.A., Kupfer, H., Sciaky, N., and Kupfer, A. (1998). Three-dimensional segregation of supramolecular activation clusters in T cells. Nature *395*, 82–86.

Oancea, E., Teruel, M.N., Quest, A.F., and Meyer, T. (1998). Green fluorescent protein (GFP)-tagged cysteine-rich domains from protein kinase C as fluorescent indicators for diacylglycerol signaling in living cells. J. Cell Biol. *140*, 485–498.

Oancea, E., Bezzerides, V.J., Greka, A., and Clapham, D.E. (2003). Mechanism of persistent protein kinase D1 translocation and activation. Dev. Cell *4*, 561–574.

Parra, M., Kasler, H., McKinsey, T.A., Olson, E.N., and Verdin, E. (2005). Protein kinase D1 phosphorylates HDAC7 and induces its nuclear export after T-cell receptor activation. J. Biol. Chem. *280*, 13762–13770.

Pettitt, T.R., Martin, A., Horton, T., Liossis, C., Lord, J.M., and Wakelam, M.J. (1997). Diacylglycerol and phosphatidate generated by phospholipases C and D, respectively, have distinct fatty acid compositions and functions. Phospholipase D-derived diacylglycerol does not activate protein kinase C in porcine aortic endothelial cells. J. Biol. Chem. *272*, 17354–17359.

Prestle, J., Pfizenmaier, K., Brenner, J., and Johannes, F.J. (1996). Protein kinase C mu is located at the Golgi compartment. J. Cell Biol. *134*, 1401–1410.

Prigozhina, N.L., and Waterman-Storer, C.M. (2004). Protein kinase D-mediated anterograde membrane trafficking is required for fibroblast motility. Curr. Biol. *14*, 88–98.

Qi, S.Y., Groves, J.T., and Chakraborty, A.K. (2001). Synaptic pattern formation during cellular recognition. Proc. Natl. Acad. Sci. USA *98*, 6548–6553.

Rey, O., and Rozengurt, E. (2001). Protein kinase D interacts with Golgi via its cysteine-rich domain. Biochem. Biophys. Res. Commun. *287*, 21–26.

Rey, O., Sinnett-Smith, J., Zhukova, E., and Rozengurt, E. (2001a). Regulated nucleocytoplasmic transport of protein kinase D in response to G protein-coupled receptor activation. J. Biol. Chem. *276*, 49228–49235.

Rey, O., Young, S.H., Cantrell, D., and Rozengurt, E. (2001b). Rapid protein kinase D translocation in response to G protein-coupled receptor activation. Dependence on protein kinase C. J. Biol. Chem. *276*, 32616–32626.

Rey, O., Yuan, J., Young, S.H., and Rozengurt, E. (2003). Protein kinase Cnu /PKD3 nuclear localization, catalytic activation and intracellular redistribution in response to G protein-coupled receptor agonists. J. Biol. Chem. *278*, 23773–23785.

Rozengurt, E., Rey, O., and Waldron, R.T. (2005). Protein kinase D signaling. J. Biol. Chem. *280*, 13205–13208.

Sanjuan, M.A., Pradet-Balade, B., Jones, D.R., Martinez, A.C., Stone, J.C., Garcia-Sanz, J.A., and Merida, I. (2003). T cell activation in vivo targets diacylglycerol kinase alpha to the membrane: a novel mechanism for Ras attenuation. J. Immunol. *170*, 2877–2883.

Spitaler, M., and Cantrell, D.A. (2004). Protein kinase C and beyond. Nat. Immunol. *5*, 785–790.

Tan, S.L., and Parker, P.J. (2003). Emerging and diverse roles of protein kinase C in immune cell signalling. Biochem. J. *376*, 545–552.

van Leeuwen, J.E., and Samelson, L.E. (1999). T cell antigen-receptor signal transduction. Curr. Opin. Immunol. *11*, 242–248.

Vega, R.B., Harrison, B.C., Meadows, E., Roberts, C.R., Papst, P.J., Olson, E.N., and McKinsey, T.A. (2004). Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. Mol. Cell. Biol. *24*, 8374–8385.

Villalba, M., Bi, K., Hu, J., Altman, Y., Bushway, P., Reits, E., Neefjes, J., Baier, G., Abraham, R.T., and Altman, A. (2002). Translocation of PKC-θ in T cells is mediated by a nonconventional, PI3-K- and Vav-dependent pathway, but does not absolutely require phospholipase C. J. Cell Biol. *157*, 253–263.

Wood, C.D., Marklund, U., and Cantrell, D.A. (2005). Dual phospholipase C/diacylglycerol requirement for protein kinase D1 activation in lymphocytes. J. Biol. Chem. *280*, 6245–6251.

Yeaman, C., Ayala, M.I., Wright, J.R., Bard, F., Bossard, C., Ang, A., Maeda, Y., Seufferlein, T., Mellman, I., Nelson, W.J., and Malhotra, V. (2004). Protein kinase D regulates basolateral membrane protein exit from trans-Golgi network. Nat. Cell Biol. *6*, 106–112.

Zhong, X.P., Hainey, E.A., Olenchock, B.A., Jordan, M.S., Maltzman, J.S., Nichols, K.E., Shen, H., and Koretzky, G.A. (2003). Enhanced T cell responses due to diacylglycerol kinase ζ deficiency. Nat. Immunol. *4*, 882–890.