Translocation of PKCθ in T cells is mediated by a nonconventional, PI3-K– and Vav-dependent pathway, but does not absolutely require phospholipase C

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PKCθ plays an essential role in activation of mature T cells via stimulation of AP-1 and NF-κB, and is known to selectively translocate to the immunological synapse in antigen-stimulated T cells. Recently, we reported that a Vav/Rac pathway which depends on actin cytoskeleton reorganization mediates selective recruitment of PKCθ to the membrane or cytoskeleton and its catalytic activation by anti-CD3/CD28 costimulation. Because this pathway acted selectively on PKCθ, we addressed here the question of whether the translocation and activation of PKCθ in T cells is regulated by a unique pathway distinct from the conventional mechanism for PKC activation, i.e., PLC-mediated production of DAG. Using three independent approaches, i.e., a selective PLC inhibitor, a PLCγ1-deficient T cell line, or a dominant negative PLCγ1 mutant, we demonstrate that CD3/CD28-induced membrane recruitment and COOH-terminal phosphorylation of PKCθ are largely independent of PLC. In contrast, the same inhibitory strategies blocked the membrane translocation of PKCα. Membrane or lipid raft recruitment of PKCθ (but not PKCα) was absent in T cells treated with phosphatidylinositol 3-kinase (PI3-K) inhibitors or in Vav-deficient T cells, and was enhanced by constitutively active PI3-K. 3-phosphoinositide-dependent kinase-1 (PDK1) also upregulated the membrane translocation of PKCθ, but did not associate with it. These results provide evidence that a nonconventional PI3-K– and Vav-dependent pathway mediates the selective membrane recruitment and, possibly, activation of PKCθ in T cells.

Introduction

Members of the PKC family play an important role in T cell activation (Altman et al., 1990). T cells express several members of the PKC family, but the relative contribution of distinct T cell–expressed PKC enzymes to T cell receptor (TCR)*/CD28-initiated signaling cascade is not well understood. However, recent work revealed that at least one Ca^{2+}-independent PKC, PKCθ, which is selectively expressed in T cells, muscle, and a few other tissues (Baier et al., 1993), plays an important role in mature T cell activation (Altman et al., 2000; Isakov and Altman, 2002). Thus, PKCθ activates AP-1 (Baier-Bitterlich et al., 1996) and NF-κB (Coudronniere et al., 2000; Lin et al., 2000) and, accordingly, receptor-induced AP-1 and NF-κB activation is blocked in peripheral T cells from PKCθ knockout mice (Sun et al., 2000). Second, engagement of antigen-specific T cells by antigen-presenting cells (APCs) leads to a rapid, stable, and high-stoichiometry localization of PKCθ, but not other T cell-expressed PKCs, to the T cell-APC contact site (Monks et al., 1997). This contact site has recently been termed the supramolecular activation complex (SMAC) (Monks et al., 1998) or the immunological synapse (IS)

(Grakoui et al., 1999). This clustering correlates with the catalytic activation of PKCθ, and it only occurs upon productive activation of T cells (Monks et al., 1997). PKCθ also positively regulates expression of the activation antigen, CD69, which is expressed in subsets of developing thymocytes and in activated T cells (Sun et al., 2000; Villalba et al., 2000a).

The selective mechanism that recruits PKCθ to the SMAC/IS during antigen stimulation remains elusive. In this regard, we found recently that Vav and Rac selectively promote the membrane and cytoskeleton translocation of PKCθ, and mediate its enzymatic activation by CD3/CD28 costimulation in a process that depends on actin cytoskeleton reorganization (Villalba et al., 2000a). A similar pathway mediates the antigen-induced translocation of PKCθ into lipid rafts (Bi et al., 2001; Villalba et al., 2001). Similarly, recent

reports indicate functional cooperation between Vav and PKCθ in several T cell signaling pathways (Dienz et al., 2000; Hehner et al., 2000; Moller et al., 2001) and with the finding that Vav is essential for actin polymerization and TCR cap formation after TCR/CD3 ligation (Fischer et al., 1998; Holsinger et al., 1998; Wülfing et al., 2000). Because this effect was specific for PKCθ (Villalba et al., 2000a), we hypothesized that it may represent a novel mechanism, which is independent on the conventional PKC activation pathway mediated by phospholipase C-γ1 (PLCγ1). In this pathway, TCR-mediated tyrosine phosphorylation and subsequent activation of PLCγ1 (Granja et al., 1991; Park et al., 1991; Secrist et al., 1991; Weiss et al., 1991) lead to hydrolysis of inositol phospholipids and production of the second messenger, DAG. Membrane-associated DAG is an essential cofactor that binds, recruits, and subsequently activates
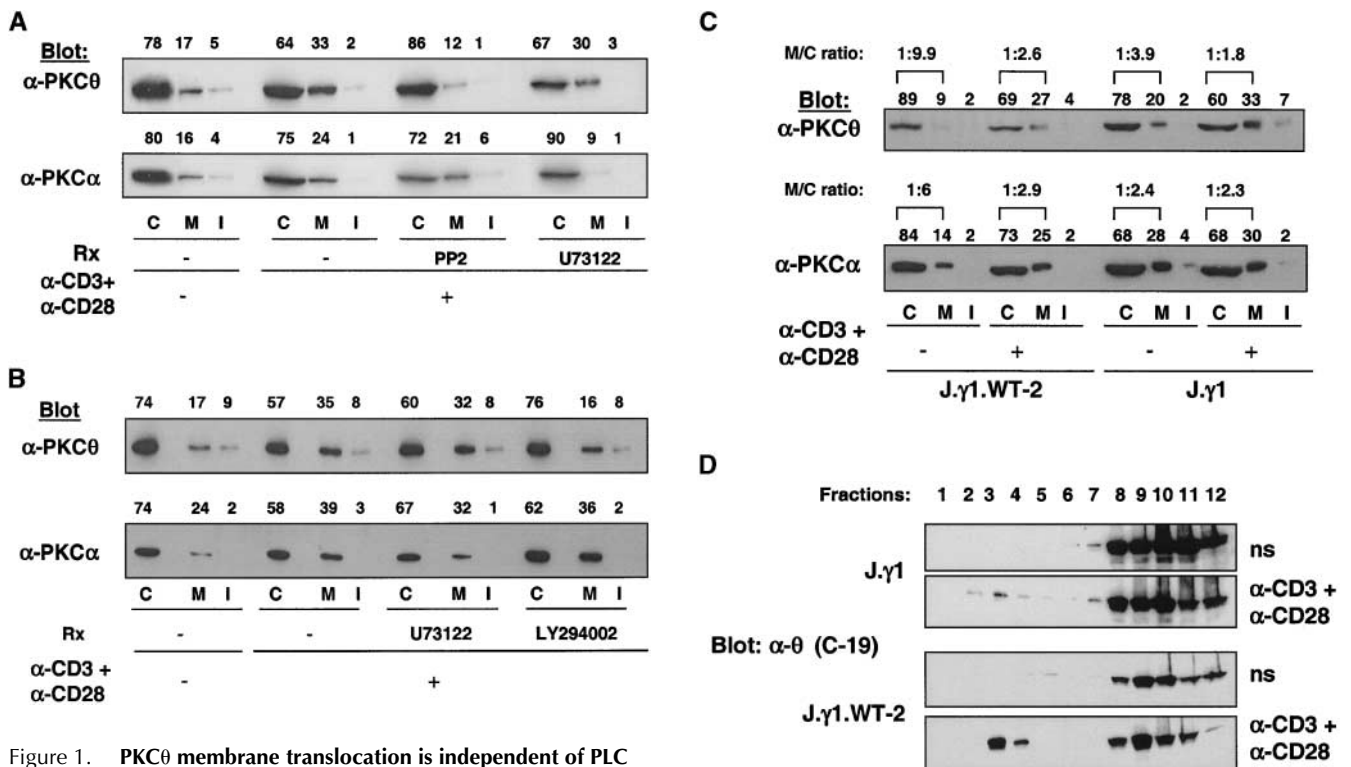
Figure 1. **PKCθ membrane translocation is independent of PLC activity.** (A) Jurkat T cells (10^6) were stimulated with anti-CD3 plus anti-CD28 antibodies for 5 min. Aliquots of the cells were preincubated for 1 h with U73122 (10 μM) or with PP2 (10 μM). Cytosol (C), membrane (M), and detergent-insoluble (I) fractions were prepared, identical cell equivalents were resolved by SDS-PAGE, and the expression of PKCθ and PKCα in each fraction was determined by immunoblotting with specific antibodies. (B) Activated human peripheral blood T cells (5×10^6) were deprived of anti-CD3 antibody for 36 h, and then restimulated with anti-CD3 plus anti-CD28 antibodies for 10 min. Aliquots of the cells were preincubated for one hr with U73122 (10 μM) or LY294002 (50 μM). Subcellular fractions were prepared and analyzed as in A. (C) J.γ1 (a PLCγ1-deficient Jurkat cell line) or J.γ1.WT-2 (PLCγ1-reconstituted J.γ1) cells were stimulated and analyzed as in A. These results are representative of three similar experiments. The membrane-to-cytosol (M/C) ratio of PKC expression in each group is displayed. In A, B, and C, the numbers above the autoradiograms represent the percentage of PKCθ or -α present in each fraction (C + M + I = 100% for each group of cells), as determined by NIH Image scanning densitometry. (D) PKCθ translocation to lipid rafts is present in PLCγ1-deficient Jurkat cells. J.γ1 or J.γ1.WT-2 (20×10^6) were left unstimulated (ns) or stimulated with anti-CD3 plus anti-CD28 antibodies. The cells were lysed and the detergent-insoluble fractions were separated from the soluble fractions. The distribution of PKCθ in each fraction was determined by immunoblotting with a specific antibody. (E) The same blot was stripped and blotted with a PLCγ1-specific antibody.

Ca^{2+}-dependent conventional PKCs (cPKCs) and Ca^{2+}-independent novel PKCs (nPKCs) in the plasma membrane (Nishizuka, 1995; Irvin et al., 2000; Zhang et al., 2000). PLCγ1 plays an important role in T cell activation, as T cells expressing a LAT mutant, which cannot recruit and activate PLCγ1, are deficient in several downstream signaling events, including Ca^{2+} mobilization and activation of the Ras/ERK pathway and NFAT (Irvin et al., 2000; Zhang et al., 2000). Similarly, a PLCγ1-deficient T cell line was recently found to display severe activation defects (Irvin et al., 2000).

In the present work, we examined the role of PLCγ1 in the membrane and lipid raft recruitment of PKCθ and its catalytic activation in T cells. Using three independent approaches to deplete or inhibit cellular PLCγ1 activity, we demonstrate that the membrane recruitment and activation of PKCθ (but not PKCα) are independent of PLCγ1. We further show that this mechanism involves Vav, phosphatidylinositol 3-kinase (PI3-K), and, indirectly, 3-phosphoinositide-dependent kinase-1 (PDK1). These results support the existence of a novel mechanism, which plays a role in the selective TCR-induced activation of PKCθ and, potentially, its recruitment to the T cell synapse.

Results

PLC-inhibiting strategies fail to block membrane recruitment and phosphorylation of PKCθ

To determine whether the reported Vav/Rac-mediated recruitment of PKCθ to the T cell membrane/cytoskeleton and its activation (Villalba et al., 2000a) are strictly dependent on activation of PLCγ1, we initially examined the effects of U73122 on the anti–CD3/CD28-induced translocation of PKCθ (or, for comparison, PKCα). This compound inhibits agonist-induced activation of PLC and the subsequent hydrolysis of inositol phospholipids in different cell types (Wang et al., 1994), including in TCR-stimulated T cells (Vassilopoulos et al., 1995). Combined anti-CD3/CD28 stimulation induced translocation of both PKC enzymes to the membrane, as evidenced by the approximately twofold increase in membrane expression of immunoreactive PKC (Fig. 1 A). As expected, U73122 pretreatment abolished the membrane translocation of PKCα and, in fact, even reduced its membrane expression below the basal level in unstimulated cells (Fig. 1 A). However, surprisingly, U73122 only minimally reduced the membrane translocation of PKCθ. As an additional control for the effectiveness of U71322 pretreatment, it also blocked the increase in intracellular calcium concentration induced by an anti-CD3 antibody (unpublished data). Conversely, PP2, an inhibitor specific for Src-family kinases, prevented the membrane translocation of PKCθ, but had only a minimal effect on PKCα translocation.

Similar results were obtained using activated human peripheral blood T cells. Thus, U73122 inhibited the anti–CD3/CD28-induced PKCα translocation, but had no significant effect on PKCθ translocation (Fig. 1 B). On the other hand, the PI3-K inhibitors LY294002 (Fig. 1 B) or wortmannin (unpublished data) essentially blocked the translocation of PKCθ, but only had a minimal effect on PKCα.

Next, we compared the receptor-induced membrane translocation of PKCθ or PKCα in J.γ1, a PLCγ1-deficient cell line, versus J.γ1.WT-2, a PLCγ1-reconstituted cell line derived from this mutant (Irvin et al., 2000). In the J.γ1 cells, anti-CD3 plus anti-CD28 stimulation still induced PKCθ, but not PKCα, translocation (Fig. 1 C). Reconstitution of J.γ1 cells with wild-type PLCγ1 (J.γ1.WT-2) restored PKCα translocation, with a minimal effect on PKCθ translocation. Calculation of the PKC membrane/cytosol expression ratio for each group of cells makes it evident that: (a) Stimulation increases the relative membrane expression of both PKCθ and α in the PLCγ1-reconstituted cells; and (b) In the PLCγ1-deficient cells, stimulation still increases the relative membrane expression of PKCθ, but not PKCα.

Anti-CD3/CD28 stimulation induces a Vav/Rac-dependent (Villalba et al., 2001) PKCθ translocation to membrane lipid rafts, which also localize at the IS (Bi et al., 2001). Therefore, we wished to determine whether this lipid raft translocation of PKCθ requires PLCγ1. Detergent-insoluble glycolipid (DIG) or soluble fractions were isolated from unstimulated or anti–CD3/CD28-stimulated J.γ1 and J.γ1.WT-2 cells, and PKCθ expression in different fractions was examined by immunoblotting. As shown previously (Bi et al., 2001; Villalba et al., 2001), stimulation induced PKCθ translocation to the DIG-containing fractions (lipid rafts) in both cell lines (Fig. 1 D), albeit the distribution pattern of PKCθ among the relevant fractions (2–4) differed between the two cell lines. Nevertheless, the overall amount of PKCθ in fractions 2–4 was higher in J.γ1.WT-2 cells when compared with the PLCγ1-deficient J.γ1 cells, suggesting some role for PLCγ1. The same fractions were probed in parallel with a PLCγ1-specific antibody. As expected, the J.γ1 cells did not express detectable amounts of PLCγ1 and, in agreement with previous results (Zhang et al., 2000), stimulation induced translocation of PLCγ1 to the lipid rafts in the reconstituted (PLCγ1 wt-2) cells (Fig. 1 E).

Activation of PKC enzymes is associated with their auto- or heterophosphorylation, events that regulate the enzymatic activity (Newton, 1997; Parekh et al., 2000). Although the regulation of PKCθ localization and/or activity by phosphorylation has not been analyzed in detail, a recent study indicated that an antibody specific for phosphorylated Thr-538 in the activation loop of PKCθ reacted specifically with the active, membrane-localized fraction of PKCθ (Bauer et al., 2001). We used another antibody specific for Ser-695 in the COOH-terminal tail of PKCθ, which is a potential autophosphorylation (Keranen et al., 1995) or heterophosphorylation (Ziegler et al., 1999; Parekh et al., 2000) site based on its homology with other PKC enzymes in order to assess the role of PLCγ1 in PKC phosphorylation. This site has very recently been implicated as a positive regulatory site in PKCθ (Liu et al., 2002). As expected, this antibody did not recognize PKCθ in unstimulated T cells, even though PKCθ was readily detected by a PKCθ-specific antibody (Fig. 2, two top panels). Anti-CD3 plus anti-CD28 stimulation induced the expected translocation of PKCθ to the insoluble fraction, which represents the pooled membranes and cytoskeleton. Unlike the PKCθ-specific antibody, the phospho-PKCθ–specific antibody only recognized PKCθ from activated cells, which was exclusively associated with the in-
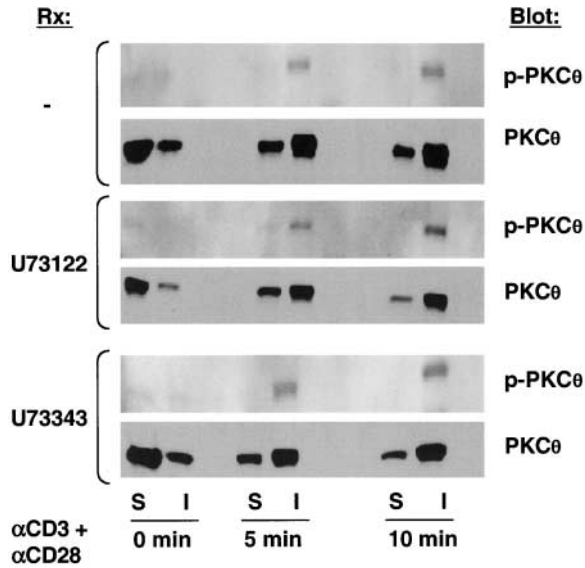
Figure 2. Inhibition of PLC does not block PKCθ COOH-terminal phosphorylation. Jurkat T cells (2 × 10^6) were left unstimulated or stimulated with anti-CD3 plus anti-CD28 antibodies (1 μg/ml each) for the indicated times. Cell aliquots were preincubated for 1 h with U73122 or U73343 (10 μM). Cytosol and insoluble fractions were prepared, resolved by SDS-PAGE, and blotted with anti-phospho-PKCθ (p-PKCθ) or anti-PKCθ antibodies. The insoluble fraction represents the combined membrane and cytoskeleton fractions, which was not further fractionated in order to minimize dephosphorylation of p-PKCθ.

soluble fraction. Importantly, pretreatment of the cells with a selective PLC inhibitor (U73122, two middle panels) or its nonfunctional analog (U73343, two bottom panels) had no significant effect on the induction and membrane translocation of phospho-PKCθ (Fig. 2).

## PLCγ1 is not required for Vav-dependent membrane clustering of PKCθ

Based on recent findings that a functional interaction between the Vav/Rac pathway and PKCθ is required for T cell activation (Dienz et al., 2000; Hehner et al., 2000; Villalba et al., 2000a; Moller et al., 2001), we considered the Vav/Rac pathway as a candidate for a selective PLCγ1-independent mechanism that recruits PKCθ to the membrane. Therefore, we next used a dominant negative PLCγ1 mutant (PLCz), which was previously found to inhibit PLCγ1-dependent functions in various cells (Chen et al., 1996), to investigate whether the Vav-induced PKCθ translocation depends on PLCγ1. Cells were cotransfected with the regulatory domain of PKCθ fused to the NH$_2$ terminus of green fluorescent protein (GFP) (Villalba et al., 2000a) plus combinations of empty vector, PLCz, and/or wild-type Vav. The intracellular localization of GFP (PKCθ) and polymerized actin (F-actin) were analyzed by confocal microscopy (Fig. 3 A)

In agreement with our previous results (Villalba et al., 2000a), either Vav overexpression or anti-CD3 stimulation induced in parallel PKCθ translocation to the membrane and F-actin accumulation, and these effects were further en-
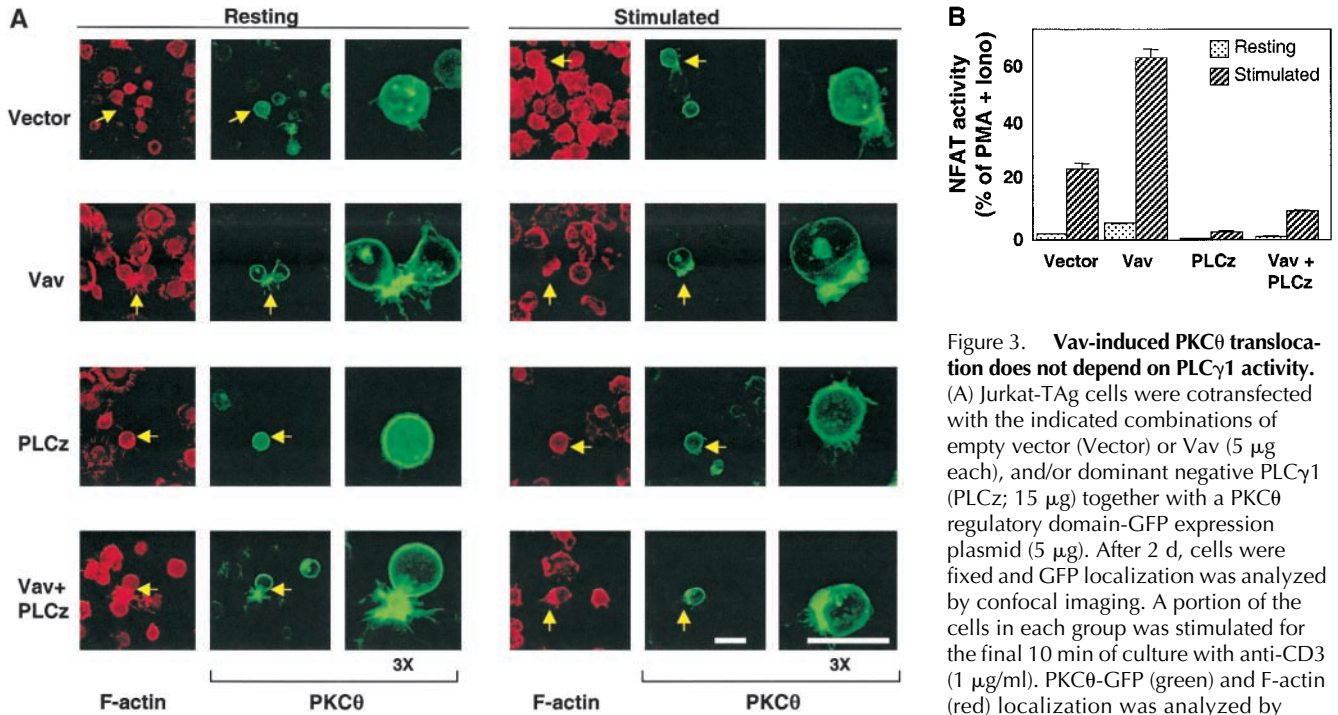
Figure 3. Vav-induced PKCθ translocation does not depend on PLCγ1 activity. (A) Jurkat-TAg cells were cotransfected with the indicated combinations of empty vector (Vector) or Vav (5 μg each), and/or dominant negative PLCγ1 (PLCz; 15 μg) together with a PKCθ regulatory domain-GFP expression plasmid (5 μg). After 2 d, cells were fixed and GFP localization was analyzed by confocal imaging. A portion of the cells in each group was stimulated for the final 10 min of culture with anti-CD3 (1 μg/ml). PKCθ-GFP (green) and F-actin (red) localization was analyzed by confocal microscopy. The right column panels in the resting or stimulated groups represent a threefold enlargement of a single cell marked with an arrow in the middle column panels. The bars in the lower right micrograph correspond to 20 μ. (B) PLCz blocks Vav- or anti-CD3–induced NFAT activation. Jurkat-TAg cells (10 × 10^6) were transfected with the indicated combinations of Vav (5 μg) and/or PLCz (15 μg) in the presence of NFAT-Luc (5 μg) and β-Gal (1.5 μg). Cells were left unstimulated or stimulated for the final 6 h of culture with anti-CD3 or with PMA (100 ng/ml) plus ionomycin (1 μg/ml). Luciferase activity was determined after 48 h of culture, and normalized to the activity of a cotransfected β-galactosidase plasmid. Data represent percentage of the response induced by PMA plus ionomycin, and are average ± standard deviation of two experiments performed in duplicate.
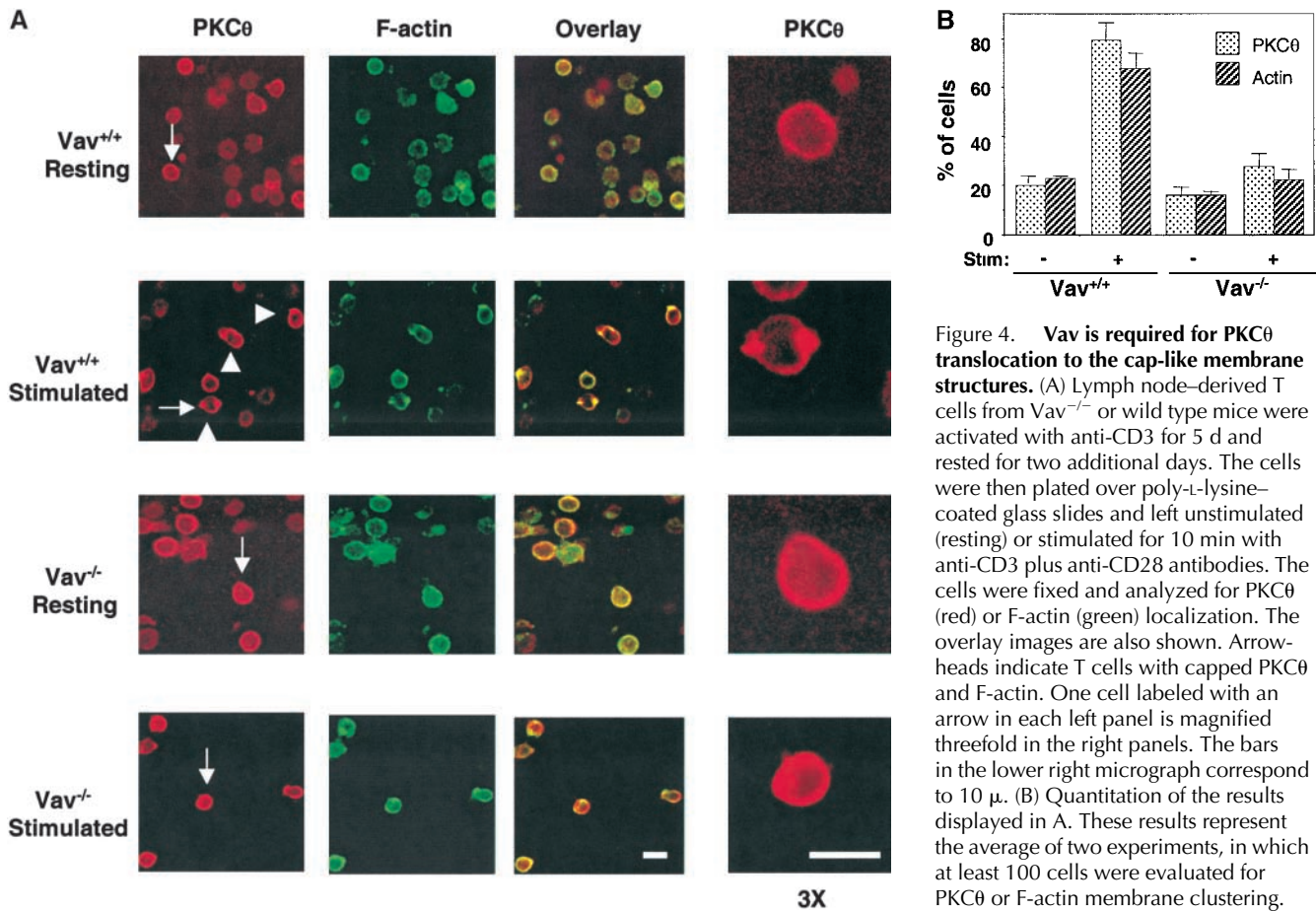
Figure 4. **Vav is required for PKCθ translocation to the cap-like membrane structures.** (A) Lymph node–derived T cells from Vav$^{-/-}$ or wild type mice were activated with anti-CD3 for 5 d and rested for two additional days. The cells were then plated over poly-L-lysine–coated glass slides and left unstimulated (resting) or stimulated for 10 min with anti-CD3 plus anti-CD28 antibodies. The cells were fixed and analyzed for PKCθ (red) or F-actin (green) localization. The overlay images are also shown. Arrowheads indicate T cells with capped PKCθ and F-actin. One cell labeled with an arrow in each left panel is magnified threefold in the right panels. The bars in the lower right micrograph correspond to 10 μ. (B) Quantitation of the results displayed in A. These results represent the average of two experiments, in which at least 100 cells were evaluated for PKCθ or F-actin membrane clustering.

hanced in anti-CD3 stimulated, Vav-transfected cells. Thus, in the latter case, a very pronounced actin capping and PKCθ colocalization, as well as F-actin–enriched, lamelipodia-like structures were observed. Coexpression of PLCz did not reduce the anti-CD3–induced membrane translocation of GFP-PKCθ, nor did it affect the membrane localization of GFP-PKCθ (or its colocalization with F-actin) in cells that were additionally transfected with Vav, and were either unstimulated or stimulated. As a control for the effectiveness of the dominant negative PLCγ1 mutant, its overexpression under similar conditions blocked the anti-CD3– and/or Vav-induced nuclear factor of activated T cells (NFAT) activation (Fig. 3 B). This dichotomy is consistent with the notion that Vav activates multiple pathways mediated by different effectors (Collins et al., 1997; Bustelo, 2000; Krawczyk et al., 2000; Villalba et al., 2000b), of which only some may depend on intact PLCγ1/Ca^{2+} signals. Taken together, the results shown above (Figs. 1–3) suggest that CD3/CD28 engagement causes membrane translocation of PKCθ via a nonconventional pathway, which appears to be, at least in part, PLCγ1-independent.

Defective membrane translocation of PKCθ in Vav-deficient primary T cells

Next, we decided to study the components of the unique pathway involved in the membrane translocation of PKCθ. First, we examined the role of Vav by comparing T cells from wild-type versus Vav-deficient T cells (Fig. 4). F-actin

localization was determined in parallel. In order to expand the T cell population from the vav$^{-/-}$ mice, their lymph node cells were activated with an anti-CD3 mAb in the presence of IL-2, and then rested prior to restimulation. In T cells derived from vav$^{+/+}$ mice, combined CD3/CD28 engagement induced actin polymerization, with a tendency of F-actin to polarize in a cap-like structure in a fraction of the cells. In agreement with previous results (Fischer et al., 1998; Holsinger et al., 1998), this outcome was clearly reduced in stimulated T cells derived from vav$^{-/-}$ mice (Fig. 4 A).

Parallel analysis of endogenous PKCθ localization demonstrated that CD3/CD28 engagement induced membrane translocation of PKCθ in wild-type T cells. This membrane expression was not uniform, but rather restricted to certain parts of the membrane where the endogenous PKCθ was found in one or more cap-like structure (Fig. 4 A). An overlay of the two images clearly demonstrated substantial colocalization of F-actin and PKCθ in the stimulated T cells. This colocalization was observed in a larger fraction of the cells by comparison with the unstimulated cells. In contrast, the stimulated T cells from vav$^{-/-}$ mice did not differ significantly from their unstimulated counterparts with regard to PKCθ localization. Although some colocalization of F-actin and PKCθ was observed in these cells, it was markedly less pronounced than in the Vav-expressing T cells. This result is in agreement with our earlier finding that a dominant negative Vav mutant blocked the anti-CD3/CD28–induced

membrane translocation of PKCθ (Villalba et al., 2000a). Quantitation of these results clearly demonstrates the defect in both PKCθ and F-actin capping in the Vav-deficient T cells (Fig. 4 B).

The role of PI3-K in Vav-mediated membrane translocation of PKCθ

PI3-K–generated lipid products activate Vav and recruit it to the membrane by binding to its pleckstrin-homology (PH) domain (Han et al., 1998). Consistent with this finding, a PI3-K inhibitor blocked the membrane translocation of PKCθ in peripheral blood T cells (Fig. 1 B). Together, these findings suggest a role for PI3-K in activating the Vav pathway involved in PKCθ membrane translocation. To address this potential role, we examined the effect of a transfected membrane-targeted (constitutively active) p110 plasmid or a PI3-K inhibitor on the membrane and cytoskeleton translocation of cotransfected PKCθ in Jurkat-TAg cells. As a positive control, we cotransfected another group of cells with Vav, which induces PKCθ translocation to these subcellular compartments (Villalba et al., 2000a).

In empty vector-transfected cells, anti-CD3 stimulation induced membrane translocation of PKCθ, which was reduced by LY294002 pretreatment (Fig. 5 A, top). Similar to Vav, p110 overexpression also induced PKCθ translocation to the membrane as well as the cytoskeleton fractions in unstimulated cells, but no significant cooperation between Vav and p110 was observed; either Vav or p110 enhanced the ability of an anti-CD3 antibody to translocate PKCθ (Fig. 5 B). Expression of p110, as well as anti-CD3 stimulation, also enhanced the membrane and cytoskeleton translocation of Vav (Fig. 5 A, two bottom panels). The PI3-K inhibitor LY294002 markedly inhibited both the p110- and Vav-induced PKCθ translocation. However, it was less effective in Vav- plus p110-cotransfected cells, possibly reflecting the strong activating effect of this combined transfection and/or sufficient tyrosine kinase-mediated and PI3-K-independent Vav activation under these conditions.

Additional experiments demonstrated that a dominant negative Vav mutant, VavΔPH (Villalba et al., 2000a), blocked the membrane and cytoskeleton translocation of PKCθ induced by p110 or anti-CD3, and even reduced the basal expression of PKCθ in these compartments in unstimulated cells (Fig. 5 B). The specificity of this effect vis-à-vis p110 and receptor (CD3) stimulation is evident from the finding that VavΔPH had no effect on PMA-induced PKCθ translocation. Of interest, the majority of the transfected VavΔPH protein localized to the cytoskeleton, and this localization was not affected by p110 coexpression (Fig. 5 B, two bottom panels). This finding suggests that VavΔPH exerts its dominant negative effect by competing with endogenous Vav for binding to potential Vav targets in the cytoskeleton compartment, where Vav is translocated following activation (Fig. 5 A). In addition, combined anti-CD3/CD28–induced PKCθ translocation into the lipid rafts was also blocked by wortmannin and LY294002 (unpublished data). Taken together, these data lend further support for the notion that PI3-K functions upstream of Vav to regulate the membrane and lipid raft translocation of PKCθ (Villalba
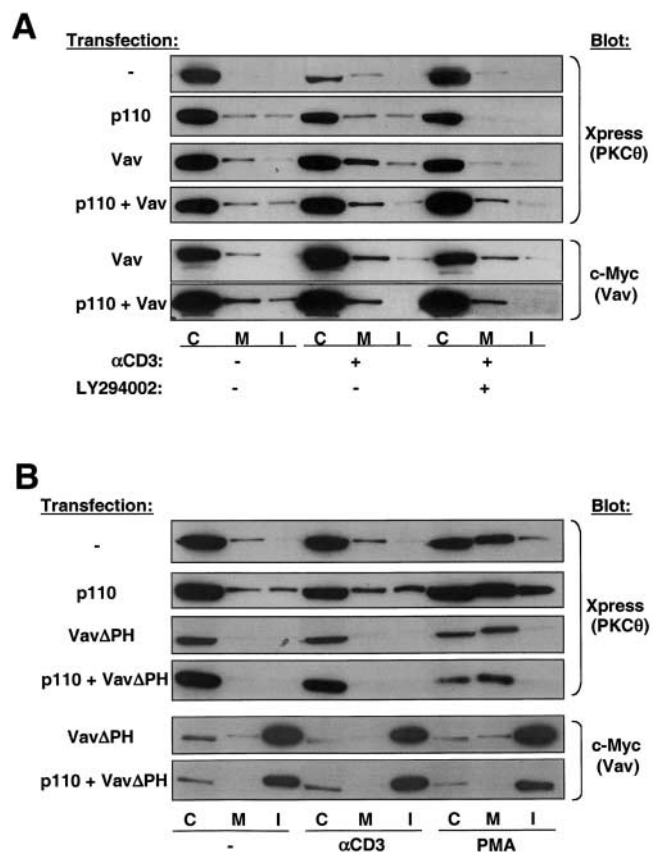
Figure 5. **Vav-mediated PKCθ translocation depends on PI3-K.** (A) Jurkat-TAg T cells were transiently cotransfected with an Xpress-tagged PKCθ expression vector plus c-Myc–tagged Vav or constitutively active PI3-K (CD2p110) expression vectors. After 40 h, the cells were left unstimulated or stimulated for 8 min with an anti-CD3 antibody (Jurkat-TAg cells do not express CD28) in the absence or presence of pretreatment with LY294002 (50 μM; 45 min). Subcellular fractionation was performed as in Fig. 1. The transfected PKCθ and Vav were detected by anti-Xpress and anti–c-Myc immunoblotting, respectively. (B) Jurkat-Tag T cells were transfected with the indicated vectors, left unstimulated or stimulated for 8 min with anti-CD3 or PMA (100 ng/ml), and analyzed as in A.

et al., 2000a, 2001). This pathway appears to be functional in Jurkat (Fig. 5) as well as normal peripheral blood T cells (Fig. 1 B).

PDK1 is indirectly involved in the membrane translocation of PKCθ

PDK1 associates with, and is responsible for, activation loop phosphorylation of different PKC enzymes (Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998; Balendran et al., 2000; Dutil and Newton, 2000; Gao et al., 2001). PDK1 and PKC need to be corecruited to the membrane through interaction with their respective membrane-localized allosteric activators in order for this phosphorylation to be efficient (Chou et al., 1998; Parekh et al., 2000; Toker and Newton, 2000; Sonnenburg et al., 2001). Calphostin C, which selectively blocks the allosteric activation of PKC by DAG, also inhibits serum-induced activation loop phosphorylation, as do PI3-K inhibitors (Parekh et al., 1999).

A

Blot:

PKCθ

PDK1

C M I C M I C M I

- | αCD3 + αCD28 | PMA

B

Transfection:

Blot:

-

PDK1

PDK1 + VavΔPH

Xpress (PKCθ)

C M I C M I C M I

- | αCD3 | LY294002

C

Transfection:

Blot:

-

PDK1

Xpress (PKCθ)

c-Myc (PDK1)

C M I C M I C M I

- | αCD3 | PMA
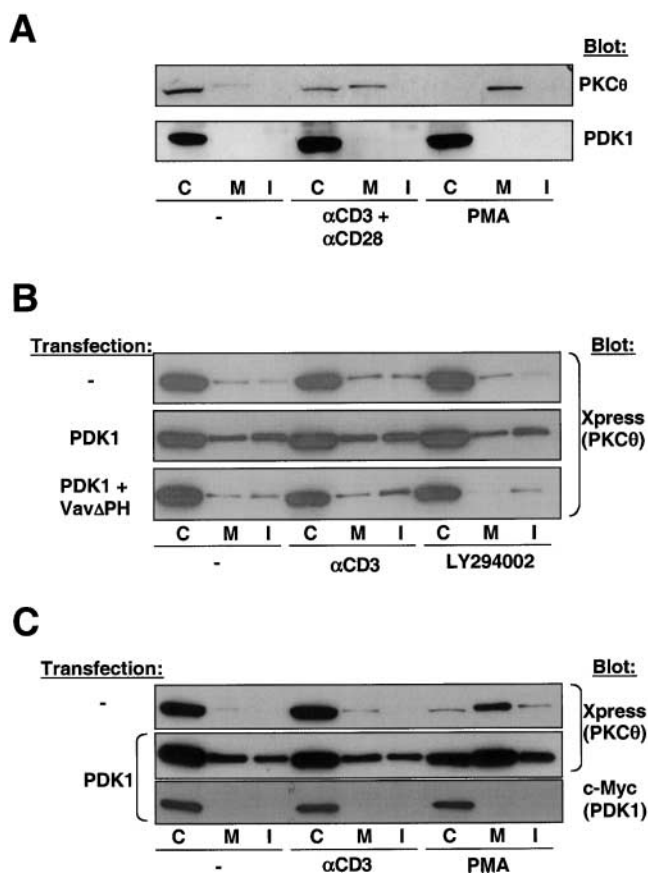
Figure 6.   **PKCθ translocation is indirectly mediated by PDK1.** (A) Jurkat E6.1 cells (10^6) were stimulated with anti-CD3 plus anti-CD28 antibodies or PMA for 8 min. Subcellular fractions were prepared and analyzed for endogenous PKCθ and PDK1 expression by immunoblotting with specific antibodies. (B) Jurkat-TAg cells were transiently cotransfected with an Xpress-tagged PKCθ expression vector plus c-Myc–tagged PDK1 or PDK1 plus VavΔPH. 40 h later, the cells were left unstimulated, or stimulated with anti-CD3 antibody. A fraction of the stimulated cells was pretreated for LY294002. Subcellular fractionation and immunoblotting were performed as in Fig. 5 and transfected PKCθ was detected by anti-Xpress immunoblotting. (C) Jurkat-TAg cells were transfected as in (B), and the cells were left unstimulated or stimulated with anti-CD3 or PMA. Transfected PKCθ and PDK1 expression in different fractions was determined by immunoblotting with the corresponding tag-specific antibodies.

These findings suggested an alternative mechanism for the membrane recruitment of PKCθ, i.e., its association with PDK1, which, by virtue of its PH domain, may localize PKCθ to the membrane. Therefore, we examined the relative localization of PDK1 and PKCθ in unstimulated or TCR-stimulated T cells.

When Jurkat T cells were stimulated with a combination of anti-CD3 plus -CD28 antibodies (or with PMA), endogenous PKCθ was clearly translocated to the membrane fraction; however, under the same conditions we could not detect similar translocation of endogenous PDK1 (Fig. 6 A), indicating that in T cells, PDK1 intracellular localization is not regulated by TCR/CD28 stimulation. To assess more directly whether PDK1 can influence the translocation of PKCθ, we cotransfected Jurkat-TAg cells with PKCθ plus

PDK1 expression vectors. PDK1 coexpression enhanced the membrane and cytoskeleton translocation of PKCθ, and this effect was only partially sensitive to a PI3-K inhibitor (Fig. 6, B and C). Interestingly, this enhanced PDK1-induced translocation of PKCθ was largely reversed by coexpression of the dominant negative (ΔPH) Vav mutant. Even under these overexpression conditions, no PDK1 was detected in the membrane and cytoskeletal fractions of the stimulated cells (Fig. 6 C). In other, functional assays we found that coexpression of PDK1 with PKCθ did not enhanced the PKCθ-induced activation of NF-κB and AP-1 reporter genes (unpublished data). These results suggest that, although PDK1 may be involved in the maturation (perhaps via activation loop phosphorylation; Bauer et al., 2001) of PKCθ in a similar manner to other PKC enzymes (Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998; Dutil and Newton, 2000; Toker and Newton, 2000), it does not directly translocate PKCθ to the membrane by associating with it in T cells.

Discussion

Membrane translocation and subsequent activation of cPKCs and nPKCs requires their conserved C1 domain, which binds the second messenger DAG formed in the inner leaflet of the plasma membrane as a result of PLC activation by various receptors (or phorbol ester pharmacophores). The importance of this event was demonstrated by findings that mutations in the C1 domain of several members of the PKC family, e.g. PKCα (a cPKC) and PKCδ (a nPKC), abolish DAG/PMA binding in vitro and/or PMA-mediated membrane translocation (Szallasi et al., 1996; Medkova and Cho, 1999). Although DAG-mediated membrane recruitment could play a role in the translocation and activation of PKCθ as well, it is difficult to explain how DAG binding alone, which is relatively nonselective, could account for the highly specific recruitment of PKCθ to the core region of the SMAC (cSMAC) or the IS. This high degree of selectivity implicates an additional undefined mechanism that either cooperates with PLC-generated DAG, or acts exclusively, to recruit PKCθ to, and activate it in, specific membrane microdomains, i.e., the cSMAC (Monks et al., 1997, 1998) or lipid rafts (Bi et al., 2001; Villalba et al., 2001). This notion is supported by our earlier work demonstrating that a Vav/Rac pathway, which involves actin cytoskeleton reorganization, mediates the membrane recruitment and activation of PKCθ (but not, e.g., PKCα) in response to TCR/CD28 engagement (Villalba et al., 2000a).

In this study we sought to further define components of the selective pathway responsible for PKCθ membrane recruitment and, furthermore, the relative importance of the conventional PLC/DAG-mediated pathway in this event. First, we used three distinct approaches, i.e., a pharmacological PLC inhibitor, a PLCγ1-deficient T cell line, and a dominant negative PLCγ1 mutant to examine the role of PLC by comparing the behavior of PKCθ to that of a representative T cell–expressed cPKC, PKCα. Each of these PLC-inhibiting strategies inhibited the membrane recruitment and/or activation of PKCα, but had, at best, a small effect on PKCθ. In addition, we demonstrate that, like Vav

(Villalba et al., 2000a), constitutively active PI3-K promotes membrane recruitment of PKCθ, and that a PI3-K inhibitor blocks this event. Furthermore, we show that a dominant negative Vav mutant blocked PI3-K (p110)-induced PKCθ translocation, suggesting that PI3-K functions either upstream of, or in parallel to, Vav to mediate PKCθ membrane translocation. The finding that recruitment of PKCθ to the cap formed by TCR/CD28 stimulation is largely absent in Vav-deficient T cells or is blocked by dominant negative Vav mutants in Vav-expressing T cells reaffirms the importance of Vav in this event.

The requirement for both Vav and PI3-K in PKCθ membrane translocation and activation most likely reflects the fact that Vav is a critical target for PI3-K in a single pathway regulating PKCθ activation in the IS. This putative mechanism is consistent with the finding that Vav is activated by PIP_3 binding to its PH domain (Han et al., 1998), a process that recruits Vav to the membrane and facilitates its catalytic activation by regulatory tyrosine phosphorylation (Aghazadeh et al., 2000). The requirement of TCR/CD28 costimulation for stable activation and membrane or lipid raft translocation of PKCθ (Coudronniere et al., 2000; Bi et al., 2001) could reflect this dual regulatory mechanism for Vav activation. Thus, PI3-K is primarily stimulated by CD28 ligation (Rudd, 1996) and tyrosine kinases such as Lck and ZAP-70 are targets for TCR signals (van Leeuwen and Samelson, 1999; Kane et al., 2000). In this regard, CD28 has been shown to colocalize with PKCθ at the IS (Monks et al., 1998), and lipid rafts, which accumulate at the IS in antigen-stimulated T cells (Bi et al., 2001), represent sites where PIP_2, the precursor of PIP_3, is formed in the membrane (Rozelle et al., 2000). Such a dual role for tyrosine kinases and PI3-K in Vav stimulation leading to PKCθ activation is also consistent with our findings that PKCθ membrane recruitment is inhibited by both Src family and PI3-K inhibitors. Finally, the finding that Vav and constitutively active PI3-K do not cooperate to enhance membrane translocation of PKCθ (Fig. 5) is also consistent with the notion that PI3-K and Vav function in a single pathway. However, we cannot formally rule out the possibility that Vav and PI3-K function in two independent pathways to promote PKCθ translocation and activation.

Although PDK1 overexpression induced prominent translocation of PKCθ to the membrane and to the cytoskeleton (Fig. 6), it itself did not undergo detectable membrane translocation upon T cell activation, even when overexpressed in T cells. These findings strongly suggest that direct association of PKCθ with PDK1 does not occur in stimulated T cells and, therefore, most likely cannot account for the inducible membrane translocation of PKCθ. Furthermore, if PDK1 associated with PKCθ, PDK1 overexpression in the cytosol would be expected to retain PKCθ in the cytosol and, thus, inhibit its anti-CD3–induced translocation to the membrane, but we actually observed the opposite result. Thus, PDK1 may play an indirect role in the membrane translocation of PKCθ, perhaps reflecting its ability to phosphorylate PKCθ and induce its maturation, as demonstrated for other members of the PKC family (Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998; Dutil and Newton,

2000; Toker and Newton, 2000). This effect appeared to be partially PI3-K–independent, consistent with a recent report (Sonnenburg et al., 2001). However, the details of this indirect effect remain to be determined. At any rate, our finding that PDK1 does not increase PKCθ-induced NF-κB or AP-1 activity (unpublished data) indicates that PDK1-mediated PKCθ translocation is not sufficient to render it functional. Finally, the ability of dominant negative Vav to inhibit PDK1-induced PKCθ translocation suggests that Vav may function downstream of PDK1. However, the two could function in separate pathways, a notion supported by the finding that, unlike Vav (Dienz et al., 2000; Hehner et al., 2000), PDK1 did not cooperate with PKCθ to activate NF-κB (unpublished data).

Our results do not completely rule out a requirement for DAG binding to the PKCθ C1 domain in initiating its membrane binding and activation. It is possible that some residual level of basal DAG that remains even under conditions of blocked PLC activity is sufficient to initiate PKCθ membrane binding. Albeit not sufficient for further recruitment of PKCθ to specific membrane compartments such as the IS or lipid rafts, it may facilitate the interaction of PKCθ with membrane or cytoskeletal component required for translocation of PKCθ to the cSMAC and its full activation. Such a component could be some membrane-localized protein kinase that transphosphorylates PKCθ or an adapter/scaffold protein that recruits it to specific membrane microdomains (Monks et al., 1997, 1998) or lipid rafts (Bi et al., 2001; Villalba et al., 2001). However, even if such a cooperative binding-activation mechanism exists, we still conclude that, unlike other PKCs, activated PLC and its lipid second messengers are not absolutely essential for PKCθ IS translocation and activation.

In summary, our study defines a Vav-, PI3-K– and, indirectly PDK1-dependent pathway(s), which selectively regulates the IS recruitment and activation of PKCθ in T cells. Thus, in addition to the conventional PLC/DAG-dependent pathway, the TCR/CD28 receptor system governs at least one additional pathway that positively regulates PKC function. Ongoing studies will define in more detail the molecular basis of this novel pathway.

Materials and methods

Antibodies and reagents

Rabbit (C-18) or goat (C-19) anti-PKCθ, goat PDK1, and rabbit anti-PLCγ1 polyclonal antibodies were obtained from Santa Cruz Biotechnology. PKCθ- or α-specific mAbs were obtained from Transduction Laboratories. The anti–human CD3 mAb (OKT3), was purified as previously described (Villalba et al., 1999). The anti–human CD28 mAb was from Pharmingen. The anti–mouse CD3 and CD28 mAbs were a gift from Dr. M. Croft (La Jolla Institute for Allergy and Immunology, San Diego, CA). The anti–human Vav mAb was from Upstate Biotechnology. Donkey anti–rabbit or sheep anti–mouse IgG antibodies were obtained from Amersham Pharmacia Biotech. LY294002, wortmannin, PP2, U73122, and U73343 were obtained from Calbiochem. All other reagents were obtained from Sigma-Aldrich. $Vav^{-/-}$ mice were a gift from Dr. V. Tybulewicz (National Institute for Medical Research, London, UK) (Turner et al., 1997; Costello et al., 1999). An anti–phospho-PKCθ antibody was generated by immunizing rabbits with a synthetic phosphopeptide corresponding to the sequence surrounding pSer-695 of PKCθ. The homologous residue in other PKC enzymes is auto phosphorylated during activation of the enzyme (Keranen et al., 1995).

Plasmids

The c-Myc–tagged Vav and VavΔPH expression plasmids in the pEF vector, an expression vector encoding the regulatory domain of PKCθ fused to the NH$_2$ terminus of GFP, Xpress-tagged PKCθ, and the luciferase reporter gene plasmid driven by synthetic NFAT sites derived from the IL-2 promoter have been described (Villalba et al., 2000a). An HA-tagged, dominant negative PLCγ1 mutant (PLCz) was a gift from Drs. Y. Abassi and K. Vuori (the Burnham Institute, San Diego, CA). This plasmid encodes the tandem SH2-SH2-SH3 domains of PLCγ1 (Chen et al., 1996). A constitutively active PI3-K plasmid (CD2p110) in the form of membrane targeted PI3-K catalytic subunit (Reif et al., 1996) was provided by Dr. D. Cantrell (Imperial cancer Research Fund, London, England). A c-Myc–tagged PDK1 construct (Chou et al., 1998) was provided by Dr. Toshi Kawakami (La Jolla Institute for Allergy and Immunology, San Diego, CA). As control for transfection efficiencies, a β-galactosidase (β-gal) expression plasmid in the pEF vector was used (Villalba et al., 2000a).

Cell culture and transfection

Jurkat T cell lines were grown in RPMI-1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, MEM nonessential amino acid solution (Life Technologies) and 100 U/ml each of penicillin G and streptomycin. Cells in a logarithmic growth phase were transfected with the indicated amounts of plasmid DNAs by electroporation as described (Villalba et al., 1999, 2000a). Human peripheral blood mononuclear cells were prepared from healthy volunteers by Ficoll-Hypaque centrifugation. Cells were stimulated with an activating anti-CD3 mAb (OKT3; 1 μg/ml) plus recombinant human IL-2 (20 U/ml) for 5 d, and then deprived of OKT3 and IL-2 36 h prior to restimulation. Mouse T cells were obtained from lymph nodes of Vav$^{-/-}$ or normal littermate mice, and purified on mouse T cell enrichment columns (R&D Systems). The cells were activated and rested as above, except an anti–mouse CD3 mAb (2C11-145; 1 μg/ml) was used.

Luciferase and β-gal assays

Transfected Jurkat-TAg cells were harvested after 2 d, washed twice with PBS, and lysed. Luciferase or β-gal activities in cell extracts were determined as described (Villalba et al., 1999). The results are expressed as arbitrary luciferase units per arbitrary β-gal units. All experiments were performed in duplicate, and were repeated several times with similar results.

Subcellular fractionation

Subcellular fractionation of Jurkat T cells or peripheral blood mononuclear cells was performed as previously described (Villalba et al., 2000a). Briefly, Jurkat T cells were resuspended in ice-cold hypotonic lysis buffer, and incubated on ice for 15 min. The cells were transferred to a 1-ml syringe, and sheared by passing them five times through a 30-gauge needle. The lysates were centrifuged at 200 g for 10 min to remove nuclei and cell debris, the supernatant was collected, and centrifuged at 13,000 g for 60 min at 4°C. The supernatant (cytosol) was collected, and the pellet was resuspended in lysis buffer, vortexed for 5 min at 4°C, and centrifuged again at 13,000 g for 60 min at 4°C. The supernatant representing the particulate (membrane) fraction was saved, and the detergent-insoluble fraction (cytoskeleton) was resuspended in 1% SDS in water. Each fraction was then diluted to with Laemmli buffer, and identical cell equivalents separated by SDS-PAGE. The subcellular fractionation of activated human PBLs was similar. However, due to their small size, cells were incubated in hypotonic buffer lysis buffer in the presence of two drops of Polybead-polystyrene 4.5 micron microspheres (Polysciences, Inc.) with constant shaking in order to facilitate their disruption. In some experiments (Fig. 3), fractionation was not continued beyond isolation of the soluble (cytosol) and insoluble (membrane plus cytoskeleton) fractions in order to minimize dephosphorylation of PKCθ.

Purification of DIG fractions

Detergent-insoluble and soluble fractions were separated as described previously (Zhang et al., 1998; Bi et al., 2001) with some modifications. Briefly, Jurkat T cells (20 × 10^6) were lysed in 1 ml MNE buffer (25 mM MES, pH 6.5, 150 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 1 mM sodium orthovanadate and 10 μg/ml protease inhibitors) containing 1% Triton X-100 for 20 min on ice and dounced 15 times. Samples were centrifuged at 1,000 g for 10 min at 4°C. The supernatants were then mixed with 1 ml 80% sucrose and transferred to Beckman ultracentrifuge tubes. 2 ml of 30% sucrose followed by 1 ml of 5% sucrose in MNE buffer were overlaid. Samples were subjected to ultracentrifugation (200,000 g) for 18 h at 4°C in a Beckman SW50Ti rotor. 12 fractions were collected from the top of the gradient. Proteins from each fraction were TCA precipitated before separation by 10% SDS-PAGE.

Immunofluorescence and confocal microscopy

Jurkat cells were incubated with or without 1 μg/ml each of anti-CD3 and anti-CD28 mAbs for 10 min over poly-L-lysine–treated microscope slides at 37°C. Cells were then fixed for 20 min with 3.7% paraformaldehyde at room temperature, permeabilized for 2 min with 0.1% Triton X-100 in PBS, blocked for 15 min with 1% BSA in PBS, and then stained with phalloidin-TRITC (Sigma-Aldrich) for 30 min. After washing four times with 1% BSA in PBS, the cells were mounted using a drop of Aqua-Poly/mount (Polysciences). Samples were viewed with a Plan-Apochromat 63× lens on a Nikon microscope. Images were taken using BIORAD MRC 1024 laser scanning confocal imaging system.

Activated mouse T cells were similarly incubated over poly-L-lysine–treated microscope slides coated or not with 5 μg/ml of anti–mouse-CD3 plus-CD28 antibodies in Tris 50 mM, pH 9, for 1 h at 37°C, followed by 4 h at 4° C. Cells were then fixed and permeabilized as described above, and stained with a polyclonal anti-PKCθ antibody (C-18) for 1 h. The cells were washed with 1% BSA in PBS, and then incubated with a secondary sheep anti–mouse IgG antibody coupled with Alexa 594 (Molecular Probes) plus phalloidin-FITC. The cells were subsequently washed and processed for confocal microscopy as described above. Microsoft PowerPoint software was used to prepare digital images of gel scans and micrographs.

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