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 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 Ca2+-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., 1998) or the immunological synapse (IS)
(Grakoui et al., 1999). This clustering correlates with the catalytic activation of PKCζ, and it only occurs upon productive activation of T cells (Monks et al., 1997). PKCζ also positively regulates the 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 PKCζ 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 PKCζ, 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 PKCζ into lipid rafts (Bi et al., 2001; Villalba et al., 2001). Similarly, recent reports indicate functional cooperation between Vav and PKCζ 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 PKCζ (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-ζ1 (PLCζ1). In this pathway, TCR-mediated tyrosine phosphorylation and subsequent activation of PLCζ1 (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 PKCζ.

Figure 1. **PKCζ membrane translocation is independent of PLC activity.** (A) Jurkat T cells (10⁶) 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 PKCζ and PKCα in each fraction was determined by immunoblotting with specific antibodies. (B) Activated human peripheral blood T cells (5 × 10⁶) 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 1 h with U73122 (10 μM) or with PP2 (10 μM). Subcellular fractions were prepared and analyzed as in A. (C) J.γ1 (a PLCζ1-deficient Jurkat cell line) or J.γ1.WT-2 (PLCζ1-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 PKCζ or α-ζ present in each fraction (C + M + I = 100% for each group of cells), as determined by NIH Image scanning densitometry. (D) PKCζ translocation to lipid rafts is present in PLCζ1-deficient Jurkat cells. J.γ1 or J.γ1.WT-2 (20 × 10⁶) 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 PKCζ in each fraction was determined by immunoblotting with a specific antibody. (E) The same blot was stripped and blotted with a PLCζ1-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). PLC\(\gamma\)1 plays an important role in T cell activation, as T cells expressing a LAT mutant, which cannot recruit and activate PLC\(\gamma\)1, 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 PLC\(\gamma\)1-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 PLC\(\gamma\)1 in the membrane and lipid raft recruitment of PKC\(\theta\) and its catalytic activation in T cells. Using three independent approaches to deplete or inhibit cellular PLC\(\gamma\)1 activity, we demonstrate that the membrane recruitment and activation of PKC\(\theta\) (but not PKC\(\alpha\)) are independent of PLC\(\gamma\)1. 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 PKC\(\theta\) and, potentially, its recruitment to the T cell synapse.

Results
PLC-inhibiting strategies fail to block membrane recruitment and phosphorylation of PKC\(\theta\)

To determine whether the reported Vav/Rac-mediated recruitment of PKC\(\theta\) to the T cell membrane/cytoskeleton and its activation (Villalba et al., 2000a) are strictly dependent on activation of PLC\(\gamma\)1, we initially examined the effects of U73122 on the anti–CD3/CD28-induced translocation of PKC\(\theta\) (or, for comparison, PKC\(\alpha\)). 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 PKC\(\alpha\) 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 PKC\(\theta\). 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 PKC\(\theta\), but had only a minimal effect on PKC\(\alpha\).

Similar results were obtained using activated human peripheral blood T cells. Thus, U73122 inhibited the anti–CD3/CD28-induced PKC\(\theta\) translocation, but had no significant effect on PKC\(\alpha\) 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 PKC\(\theta\), but only had a minimal effect on PKC\(\alpha\).

Next, we compared the receptor-induced membrane translocation of PKC\(\theta\) or PKC\(\alpha\) in J-\(\gamma\)1, a PLC\(\gamma\)1-deficient cell line, versus J-\(\gamma\)1.WT-2, a PLC\(\gamma\)1-reconstituted cell line derived from this mutant (Irvin et al., 2000). In the J-\(\gamma\)1 cells, anti-CD3 plus anti-CD28 stimulation still induced PKC\(\theta\), but not PKC\(\alpha\), translocation (Fig. 1 C). Reconstitution of J-\(\gamma\)1 cells with wild-type PLC\(\gamma\)1 (J-\(\gamma\)1.WT-2) restored PKC\(\alpha\) translocation, with a minimal effect on PKC\(\theta\) 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 PKC\(\theta\) and \(\alpha\) in the PLC\(\gamma\)1-reconstituted cells; and (b) In the PLC\(\gamma\)1-deficient cells, stimulation still increases the relative membrane expression of PKC\(\theta\), but not PKC\(\alpha\).

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

Activation of PKC enzymes is associated with their autophosphorylation, events that regulate the enzymatic activity (Newton, 1997; Parekh et al., 2000). Although the regulation of PKC\(\theta\) 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 PKC\(\theta\) reacted specifically with the active, membrane-localized fraction of PKC\(\theta\) (Bauer et al., 2001). We used another antibody specific for Ser-695 in the COOH-terminal tail of PKC\(\theta\), 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 PLC\(\gamma\)1 in PKC phosphorylation. This site has very recently been implicated as a positive regulatory site in PKC\(\theta\) (Liu et al., 2002). As expected, this antibody did not recognize PKC\(\theta\) in unstimulated T cells, even though PKC\(\theta\) was readily detected by a PKC\(\theta\)-specific antibody (Fig. 2, two top panels). Anti-CD3 plus anti-CD28 stimulation induced the expected translocation of PKC\(\theta\) to the insoluble fraction, which represents the pooled membranes and cytoskeleton. Unlike the PKC\(\theta\)-specific antibody, the phospho-PKC\(\theta\)-specific antibody only recognized PKC\(\theta\) from activated cells, which was exclusively associated with the in-
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-PKC\(\alpha\) (Fig. 2).

**PLC\(\gamma\) is not required for Vav-dependent membrane clustering of PKC\(\alpha\)**

Based on recent findings that a functional interaction between the Vav/Rac pathway and PKC\(\alpha\) 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 PLC\(\gamma\)-independent mechanism that recruits PKC\(\alpha\) to the membrane. Therefore, we next used a dominant negative PLC\(\gamma\) mutant (PLC\(\gamma\)), which was previously found to inhibit PLC\(\gamma\)-dependent functions in various cells (Chen et al., 1996), to investigate whether the Vav-induced PKC\(\alpha\) translocation depends on PLC\(\gamma\). Cells were cotransfected with the regulatory domain of PKC\(\alpha\) fused to the NH\(_2\) terminus of green fluorescent protein (GFP) (Villalba et al., 2000a) plus combinations of empty vector, PLC\(\gamma\), and/or wild-type Vav. The intracellular localization of GFP (PKC\(\alpha\)) 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 PKC\(\alpha\) translocation to the membrane and F-actin accumulation, and these effects were further en-

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**Figure 2.** Inhibition of PLC does not block PKC\(\alpha\) COOH-terminal phosphorylation. Jurkat T cells (2 \(\times\) 10\(^{6}\)) were left unstimulated or stimulated with anti-CD3 plus anti-CD28 antibodies (1 \(\mu\)g/ml each) for the indicated times. Cell aliquots were preincubated for 1 h with U73122 or U73343 (10 \(\mu\)M). Cytosol and insoluble fractions were prepared, resolved by SDS-PAGE, and blotted with anti-phospho-PKC\(\alpha\) (p-PKC\(\alpha\)) or anti-PKC\(\alpha\) antibodies. The insoluble fraction represents the combined membrane and cytoskeleton fractions, which was not further fractionated in order to minimize dephosphorylation of p-PKC\(\alpha\).

**Figure 3.** Vav-induced PKC\(\alpha\) translocation does not depend on PLC\(\gamma\) activity. (A) Jurkat-TAg cells were cotransfected with the indicated combinations of empty vector (Vector) or Vav (5 \(\mu\)g each), and/or dominant negative PLC\(\gamma\) (PLC\(\gamma\); 15 \(\mu\)g) together with a PKC\(\alpha\) regulatory domain-GFP expression plasmid (5 \(\mu\)g). After 2 d, cells were fixed and GFP localization was analyzed by confocal microscopy. A portion of the cells in each group was stimulated for the final 10 min of culture with anti-CD3 (1 \(\mu\)g/ml). 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 \(\mu\). (B) PLC\(\gamma\) blocks Vav- or anti-CD3–induced NFAT activation. Jurkat-TAg cells (10 \(\times\) 10\(^{6}\)) were cotransfected with the indicated combinations of Vav (5 \(\mu\)g) and/or PLC\(\gamma\) (15 \(\mu\)g) in the presence of NFAT-Luc (5 \(\mu\)g) and \(\beta\)-Gal (1.5 \(\mu\)g). 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 \(\mu\)g/ml). Luciferase activity was determined after 48 h of culture, and normalized to the activity of a cotransfected \(\beta\)-galactosidase plasmid. Data represent percentage of the response induced by PMA plus ionomycin, and are average \(\pm\) standard deviation of two experiments performed in duplicate.
hanced in anti-CD3 stimulated, Vav-transfected cells. Thus, in the latter case, a very pronounced actin capping and PKCα colocalization, as well as F-actin–enriched, lamellipodia-like structures were observed. Coexpression of PLCz did not reduce the anti-CD3–induced membrane translocation of GFP-PKCα (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/Ca2+ signals. Taken together, the results shown above (Figs. 1–3) suggest that CD3/CD28 engagement causes membrane translocation of PKCα via a nonconventional pathway, which appears to be, at least in part, PLCγ1-independent.

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

Next, we decided to study the components of the unique pathway involved in the membrane translocation of PKCα. 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 PKCα localization demonstrated that CD3/CD28 engagement induced membrane translocation of PKCα in wild-type T cells. This membrane expression was not uniform, but rather restricted to certain parts of the membrane where the endogenous PKCα 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 PKCα 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 PKCα localization. Although some colocalization of F-actin and PKCα 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 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; Dutil 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).
PKC family, e.g. PKCα (a cPKC) and PKCθ (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, PKCθ. Each of these PLC-inhibiting strategies inhibited the membrane recruitment and/or activation of PKCθ, but had, at best, a small effect on PKCθ. In addition, we demonstrate that, like Vav

\[ PKCθ \]
The requirement of TCR/CD28 costimulation (Aghazadeh et al., 2000). The requirement of TCR/CD28 costimulation to colocalize with PKC \( \text{ζ} \) has been shown to be mediated by regulatory tyrosine phosphorylation (Samelson, 1999; Kane et al., 2000). In this regard, CD28 interaction with ZAP-70 are targets for TCR signals (van Leeuwen and Samelson, 1998), and lipid rafts, which accumulate at the IS in an antigen-stimulated T cells (Bi et al., 2001), represent sites where PIP\(_3\), the precursor of PIP\(_{3}\) raft translocation of PKC \( \text{ζ} \) (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 ligand (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 PKC \( \text{ζ} \) at the IS (Monks et al., 1998), and lipid rafts, which accumulate at the IS in an antigen-stimulated T cells (Bi et al., 2001), represent sites where PIP\(_3\), 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 PKC \( \text{ζ} \) translocation is also consistent with our findings that PKC \( \text{ζ} \) 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 PKC \( \text{ζ} \) (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 PKC \( \text{ζ} \) translocation and activation.

Although PDK1 overexpression induced prominent translocation of PKC \( \text{ζ} \) 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 PKC \( \text{ζ} \) with PDK1 does not occur in stimulated T cells and, therefore, most likely cannot account for the inducible membrane translocation of PKC \( \text{ζ} \). Furthermore, if PDK1 associated with PKC \( \text{ζ} \), PDK1 overexpression in the cytosol would be expected to retain PKC \( \text{ζ} \) 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 PKC \( \text{ζ} \), perhaps reflecting its ability to phosphorylate PKC \( \text{ζ} \) 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 PKC \( \text{ζ} \)–induced NF-κB activity (unpublished data) indicates that PDK1-mediated PKC \( \text{ζ} \) translocation is not sufficient to render it functional. Finally, the ability of dominant negative Vav to inhibit PKC \( \text{ζ} \)–induced PKC \( \text{ζ} \) 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 PKC \( \text{ζ} \) to activate NF-κB (unpublished data).

Our results do not completely rule out a requirement for DAG binding to the PKC \( \text{ζ} \) 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 PKC \( \text{ζ} \) membrane binding. Albeit not sufficient for further recruitment of PKC \( \text{ζ} \) to specific membrane compartments such as the IS or lipid rafts, it may facilitate the interaction of PKC \( \text{ζ} \) with membrane or cytoskeletal component required for translocation of PKC \( \text{ζ} \) to the cSMAC and its full activation. Such a component could be some membrane-localized protein kinase that transphosphorylates PKC \( \text{ζ} \) 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 PKC \( \text{ζ} \) 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 PKC \( \text{ζ} \) 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-PKC\( \text{ζ} \), goat PDK1, and rabbit anti-PLC\( \text{γ} \) polyclonal antibodies were obtained from Santa Cruz Biotechnology. PKC\( \varepsilon \) 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 Pharma- cius Biotech. LY294002, wortmannin, PP2, U73122, and U73343 were obtained from 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-PKC\( \text{ζ} \) antibody was generated by immunizing rabbits with a synthetic phosphopeptide corresponding to the sequence surrounding pSer-695 of PKC\( \text{ζ} \). 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 PKCδ fused to the NH2 terminus of GFP, Xpress-tagged PKCδ, 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 (PLCδ) 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., 1990) was provided by Dr. Toshi Kawakami (La Jolla, CA). A PI3-K catalytic subunit (Reif et al., 1996) was provided by Dr. D. Cantrell (Imperial cancer Research Fund, London, England). A c-Myc–tagged Vav and VavΔPH 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, MEGA 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 mixed 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 (the skeleton) was resuspended in 1% SDS in water. Each fraction was then diluted 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 Polybrene-polyethylene 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 PKCδ.

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 × 105) 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 phal-loidin-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×/1.4 oil 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-PKCδ antibody (C-18) for 1 h. The cells were washed with 1% BSA in PBS, and then incubated with a secondary sheep anti-rabbit 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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