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A Novel Functional Interaction between Vav and PKCθ Is Required for TCR-Induced T Cell Activation

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Summary

Vav and PKCθ play an early and important role in the TCR/CD28-induced stimulation of MAP kinases and activation of the IL-2 gene. Vav is also essential for actin cytoskeleton reorganization and TCR capping. Here, we report that PKCθ function was selectively required in a Vav signaling pathway that mediates the TCR/CD28-induced activation of JNK and the IL-2 gene and the upregulation of CD69 expression. Vav also promoted PKCθ translocation from the cytosol to the membrane and cytoskeleton and induced its enzymatic activation in a CD3/CD28-initiated pathway that was dependent on Rac and on actin cytoskeleton reorganization. These findings reveal that the Vav/Rac pathway promotes the recruitment of PKCθ to the T cell synapse and its activation, essential processes for T cell activation and IL-2 production.

Introduction

T cell activation induced by triggering of the antigen-specific T cell receptor (TCR)/CD3 complex in concert with costimulatory and adhesion receptors is a complex process that involves multiple enzymes, adapters, and other cellular proteins. Activation is initiated by stimulation of TCR-coupled protein tyrosine kinases (PTKs) of the Src and Syk families, which then phosphorylate various cellular substrates (reviewed in Weiss and Littman, 1994). This is followed by the recruitment and assembly of membrane signaling complexes that mediate different signal transduction pathways. These signals are relayed to the nucleus, where they induce a defined genetic program, of which the best characterized constituent is the activation of the interleukin-2 (IL-2) gene by coordinated binding of several transcription factors to the IL-2 gene promoter (reviewed in Crabtree and Clipstone, 1994; Jain et al., 1995). Recent studies have indicated that formation of the T cell synapse (Grakoui et al., 1999) and assembly of productive signaling complexes depend on reorganization of the actin cytoskeleton (reviewed in Penninger and Crabtree, 1999) and are regulated, in addition, by specialized lipid microdomains (reviewed in Xavier and Seed, 1999).

Vav (reviewed in Romero and Fischer, 1996; Collins et al., 1997; Cantrell, 1998) and protein kinase C-θ (PKCθ; reviewed in Meller et al., 1998) represent two critical enzymes in the TCR signaling machinery leading to IL-2 production. Analysis of Vav-deficient mice indicated that Vav is required for T cell development and antigen receptor-mediated T or B lymphocyte activation (Fischer et al., 1995; Zhang et al., 1995; Turner et al., 1997) as well as for TCR clustering and actin cytoskeleton reorganization (Fischer et al., 1998; Holsinger et al., 1998). Proper Vav function is also necessary for receptor-induced activation of the MAP kinase ERK and the transcription factors NFAT and NF-κB and for intact Ca^{2+} mobilization (Fischer et al., 1998; Holsinger et al., 1998; Costello et al., 1999). Consistent with these findings, Vav overexpression in T cells enhances activation of transcriptional elements in the IL-2 gene (Wu et al., 1995; Deckert et al., 1996), in particular NFAT.

PKCθ is a novel Ca^{2+}-independent PKC isoform (Baier et al., 1993) that plays an important early role in TCR-induced activation (Meller et al., 1998). Among several PKC isoforms tested, only PKCθ was capable of significantly stimulating Ras-dependent transcription from an AP-1 element in T cells (Baier-Bitterlich et al., 1996). Furthermore, PKCθ specifically cooperates with calcineurin (Cn) and plays a critical role in c-Jun N-terminal kinase (JNK) activation and induction of the IL-2 gene (Werlen et al., 1998; Ghaffari-Tabrizi et al., 1999). In addition, PKCθ selectively colocalizes with the TCR/CD3 complex in the central core of the supramolecular activation complex (SMAC) that forms at the contact region between antigen-specific T cells and antigen-presenting cells (APCs; Monks et al., 1997, 1998).

Several recent studies indirectly suggested that Vav and PKCθ display some functional similarities and/or operate in overlapping signaling pathways. For example, both signaling molecules can cooperate with a Ca^{2+} signal or with constitutively active Cn (CA-Cn) to activate JNK and the IL-2 gene (Werlen et al., 1998; Villalba et al., unpublished data); PKC activation by phorbol ester can overcome the TCR-induced signaling defects (Fischer et al., 1998; Holsinger et al., 1998) and the resistance to apoptosis (Kong et al., 1998) in Vav-deficient T cells or thymocytes, respectively. In the latter case, Vav was found to constitutively associate with PKCθ but not with other PKC isoforms (Kong et al., 1998).

These findings prompted us to study possible interactions between those two signaling proteins. Here, we provide evidence that PKCθ is a Vav effector in T cells and that the requirement for intact PKCθ function dissociates two distinct Vav signaling pathways, i.e., its ability to stimulate IL-2 gene transcription, JNK activation, and CD69 upregulation versus actin cytoskeleton reorganization.

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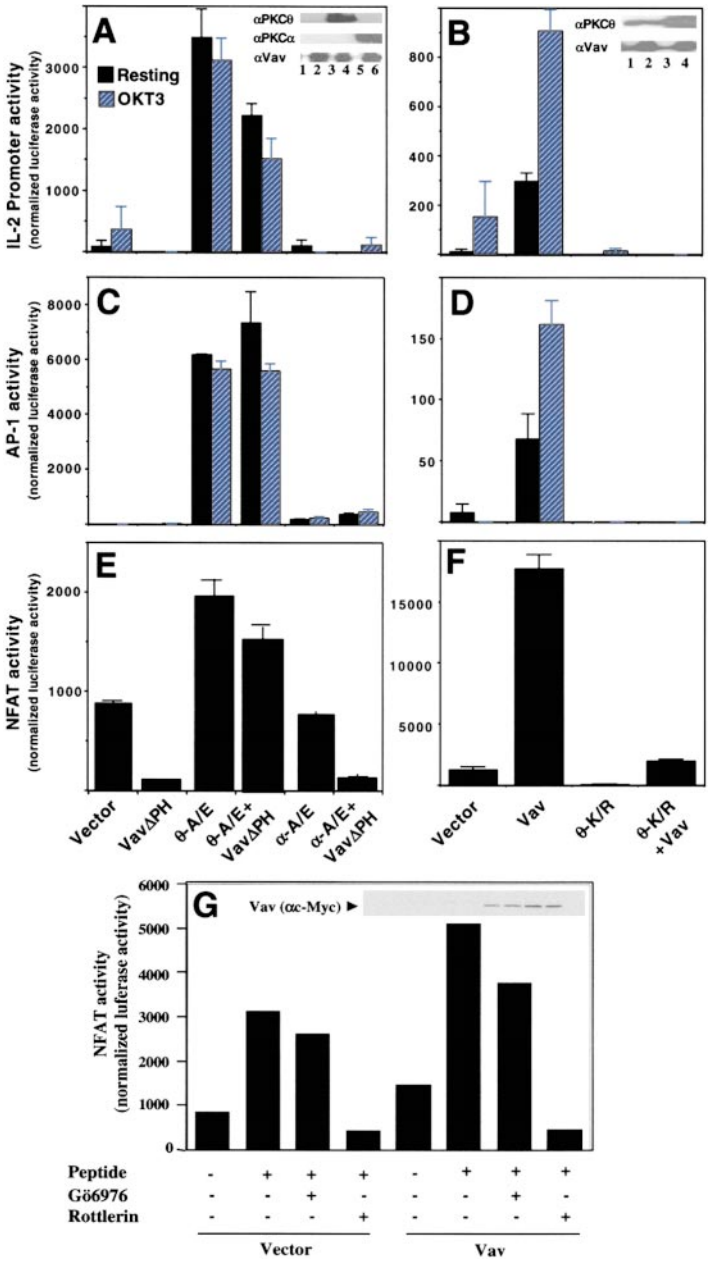

Figure 1. Interaction between Vav and PKCθ in IL-2 Promoter, AP-1, and NFAT Activation

Jurkat-TAg cells (10×10^6) were transfected with the indicated combinations of constitutively active (A/E) PKCθ or α mutants (5 μg) and/or DN Vav (ΔPH; 10 μg) (A, C, and E) or with wild-type Vav (5 μg) and/or DN (K/R) PKCθ (10 μg; B, D, and E) in the presence of an IL-2-Luc (A and B), AP-1-Luc (C and D), or NFAT-Luc (E and F) reporter plasmid (5 μg each). Cells were left unstimulated or stimulated for the final 6 hr of culture with an anti-CD3 mAb (OKT3; 1 μg/ml). Luciferase activity was determined after 48 hr of culture and normalized to the activity of a cotransfected β-galactosidase plasmid. Maximal luciferase activity (in arbitrary luciferase units) of the IL-2, AP-1, or NFAT reporter in PMA-stimulated plus ionomycin-stimulated cells was 21,800, 100,000, and 400,000 arbitrary luciferase units, respectively. OKT3-stimulated activity of the NFAT reporter was 300,000 arbitrary luciferase units. Overexpression of the transfected proteins was confirmed by immunoblotting with the corresponding PKC- or Vav-specific antibodies ([A and B], insets). The numbers under the different lanes correspond to the transfection groups shown in each panel. Equivalent Vav and PKC expression levels were also detected in the AP-1 and NFAT assays (data not shown).

(G) CH7C17 cells were transfected with the indicated combinations of empty vector, Vav, and/or NFAT reporter plasmids. After 24 hr in culture, the cells (10×10^6/group) were stimulated for an additional 6 hr with 2×10^6 mitomycin C–treated APCs alone or with APCs plus HA peptide (10 μg/ml) in the absence or presence of rottlerin (10 μM) or Gö6976 (.5 μM). Luciferase activity was quantitated as above. The inset shows the expression levels of transfected Vav.

Results

A Selective Requirement of PKCθ in Vav-Induced IL-2 Gene Activation

In order to clarify whether these two proteins function in an overlapping pathway, we evaluated the effects of transfection with combinations of active or dominant-negative (DN) mutants of PKCθ and/or Vav on the basal and/or anti-CD3-stimulated activity of an IL-2 promoter-reporter gene in transiently transfected Jurkat T cells. As shown in Figure 1A, stimulation with an anti-CD3 monoclonal antibody (mAb; OKT3) induced minimal activation of the IL-2 promoter, consistent with findings that optimal induction of this promoter requires combined stimulation with an anti-CD3 antibody plus phorbol ester (Crabtree and Clipstone, 1994; Jain et al., 1995). A constitutively active PKCθ mutant, θ-A/E, induced significant activation of the IL-2 promoter, which was similar in the absence or presence of OKT3 stimulation. This activity represented ~15% of the maximal activity induced by stimulation with phorbol ester (PMA) plus ionomycin. This effect of θ-A/E was selective since a constitutively active PKCα mutant failed to induce significant activity, even though it was properly overexpressed (Figure 1A, inset) and was more active than θ-A/E in stimulating the activity of a cotransfected ERK2 reporter (data not shown; Werlen et al., 1998). The equal expression levels of PKCθ and α were confirmed by using the corresponding epitope-tagged vectors in similar experiments (Coudronniere et al., 2000). Transient overexpression of VavΔPH, a DN Vav mutant whose pleckstrin-homology (PH) domain has been deleted (Villalba et al., unpublished data), blocked both the basal and OKT3-induced weak promoter activity in empty vector-transfected

cells. In contrast, the same Vav mutant only partially reduced the θ-A/E-induced promoter activity under either basal (30% inhibition) or stimulated (50% inhibition) conditions.

As reported earlier (Wu et al., 1995), transient Vav overexpression stimulated the basal activity of the IL-2 promoter and enhanced the anti-CD3-induced activity (Figure 1B). However, Vav was less active than θ-A/E (compare Figure 1A versus 1B). A DN PKCθ mutant (θ-K/R) blocked the promoter activity induced by OKT3 stimulation and/or transfected Vav. This effect was selective since, under similar conditions, the same θ-K/R mutant did not inhibit the ability of Vav to promote actin polymerization (Figure 4B). Immunoblotting with anti-PKCθ or -Vav antibodies confirmed the overexpression of the relevant proteins in the appropriate transfection groups (Figures 1A and 1B, insets). These findings suggest that functional PKCθ is required for TCR- and/or Vav-stimulated promoter activity and tentatively place PKCθ downstream of Vav or in an essential pathway that acts in parallel to Vav.

Similar to their effects on the IL-2 promoter, θ-A/E and Vav were each capable of activating the AP-1 (Figures 1C and 1D) or NFAT (Figures 1E and 1F) reporters, respectively. PKCθ-A/E was much more effective than Vav in inducing AP-1 activity (compare Figure 1C versus 1D) but, conversely, Vav was a more potent activator of NFAT than θ-A/E (compare Figure 1F versus 1E). As in the case of the IL-2 promoter, the stimulatory effect of θ-A/E was specific since α-A/E failed to stimulate AP-1 or NFAT and VavΔPH failed to significantly inhibit the θ-A/E-induced activities of these two reporters (Figures 1C and 1E). In contrast, the DN PKCθ mutant blocked the Vav-induced reporter activity (Figures 1D and 1F). We were unable to address in an informative manner the effect of VavΔPH on PKCθ-A/E-induced NFAT activity in activated cells since PKCθ-A/E consistently inhibited the anti-CD3-stimulated NFAT activity. The reason for this is unclear, but it may be related to the observed inhibition of Ca^{2+}-dependent NFAT activity in T cells by constitutively active Ras (Chen et al., 1996) since our previous work established a functional connection between PKCθ and Ras (Baier-Bitterlich et al., 1996).

The biological relevance of these findings was further established by using a previously characterized antigen-dependent activation system, i.e., variant Jurkat cells (CH7C17) that stably express an αβ-TCR specific for a hemagglutinin (HA)-derived peptide (Hewitt et al., 1992). Stimulation of these cells with peptide plus APCs induced activation of the NFAT reporter, which was further enhanced by transient Vav overexpression (Figure 1G). Rottlerin, a selective inhibitor of PKCθ (Villalba et al., 1999a; Coudronniere et al., 2000), blocked antigen-induced NFAT activation and reduced it to a level lower than the basal activity in unstimulated cells. In contrast, Gö6976, an inhibitor selective for Ca^{2+}-dependent PKC isoforms (Villalba et al., 1999), caused minimal inhibition of NFAT activation (Figure 1G).

Relationship between Vav and PKCθ in JNK Activation

Vav cannot efficiently activate JNK in T cells, but it can cooperate with CA-Cn to do so (Villalba et al., unpublished data). On the other hand, PKCθ is unique among

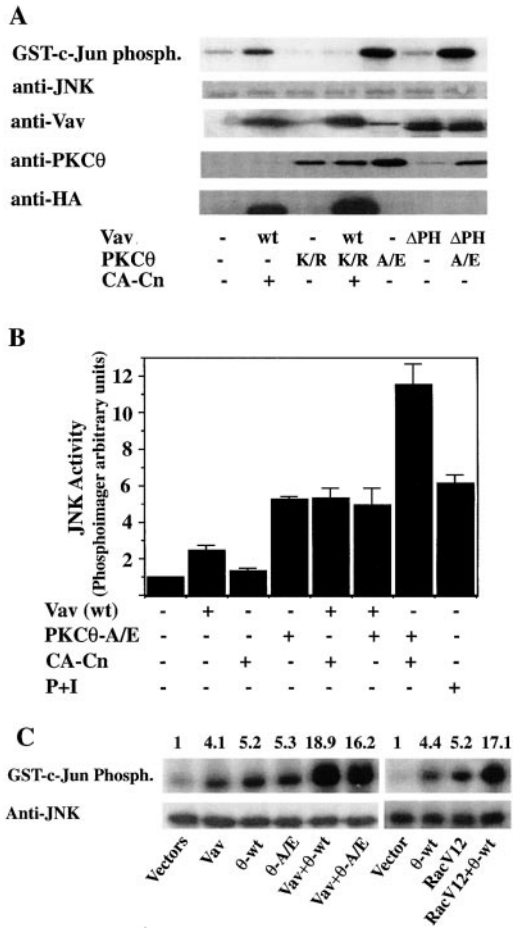

Figure 2. Interactions between Vav and PKCθ in JNK Activation

(A) PKCθ is downstream of Vav in the pathway leading to JNK activation. Jurkat-TAg cells were transfected with an HA-JNK1 plasmid (5 μg) together with CA-Cn, CA-PKCθ, and Vav or with 10 μg of DN-PKCθ or DN-Vav. After 2 days, HA-JNK was immunoprecipitated with an anti-HA antibody and JNK kinase activity was determined by an in vitro immune complex kinase assay. Under the same conditions, DN-PKCθ blocked the anti-CD3 plus CD28-induced activation of JNK (data not shown). These results are representative of three similar experiments.

(B) Vav and PKCθ do not cooperate to activate JNK in T cells. Cells were transfected and analyzed as in (A). As a control, some cells were stimulated for 15 min with PMA (100 ng/ml) plus ionomycin (1 μg/ml). Results represent the mean ± SD of three experiments.

(C) Vav and PKCθ synergize to activate JNK in nonhematopoietic cells. 293T cells (1×10^6) were transfected with the indicated plasmids plus HA-JNK1 (5 μg each). JNK kinase activity (top panel) or its expression level (bottom panel) were determined as described in (A) and (B). The numbers above each lane represent the fold activation of JNK relative to the empty vector-transfected group (=1), as determined by phosphorimager analysis.

T cell–expressed PKCs in its ability to activate JNK and to cooperate with CA-Cn (Werlen et al., 1998; Ghaffari-Tabrizi et al., 1999). Therefore, we ascertained the interrelationship between PKCθ and Vav with regard to JNK activation. Transient coexpression of Vav and CA-Cn induced significant activation of a cotransfected JNK plasmid (Figure 2A), and, moreover, this activation was completely blocked by coexpressed θ-K/R. On the other hand, θ-A/E by itself also activated JNK, but VavΔPH

failed to inhibit this effect. The same Vav mutant inhibited, however, JNK activation induced by anti-CD3 plus anti-CD28 stimulation (Villalba et al., unpublished data).

In addition, Vav or CA PKCθ cooperated with CA-Cn but not with each other to induce JNK activity (Figure 2B). This lack of cooperation between Vav and PKCθ favors the idea that PKCθ acts downstream of Vav in the same (or a partially overlapping) pathway leading to JNK activation and, furthermore, that this pathway cooperates with Ca^{2+}/Cn signals. This is consistent with the two-signal requirement for JNK activation in T cells (Su et al., 1994; Werlen et al., 1998).

Since this two-signal requirement is unique to T cells (Su et al., 1994) and neither PKCθ (Werlen et al., 1998) nor Vav (Villalba et al., unpublished data) cooperate with Cn in nonhematopoietic cells, it might be predicted that ectopic expression of Vav plus PKCθ will result in a cooperative effect in cells that lack both proteins. To test this possibility, we analyzed the effects of Vav and/or PKCθ on the activity of a cotransfected JNK reporter in heterologous 293 cells (Figure 2C). Each of these proteins stimulated the activity of JNK compared to the empty vector-transfected cells (lanes 2–4 versus lane 1). When the two were combined, synergistic effect was observed (lanes 5 and 6). No significant difference was observed between wild-type and constitutively active PKCθ, perhaps reflecting the possibility that normal PKCθ regulatory mechanisms are absent in 293 cells. Nevertheless, PKCθ activity was essential for JNK activation since the kinase-inactive θ-K/R, either alone or in combination with Vav (data not shown), failed to stimulate JNK activity in 293 cells. Similar to Vav, a constitutively active Rac mutant (RacV12) also synergized with PKCθ to activate JNK (lanes 7–10). A constitutively active Cdc42 mutant (Cdc42V12) behaved in a similar manner (data not shown).

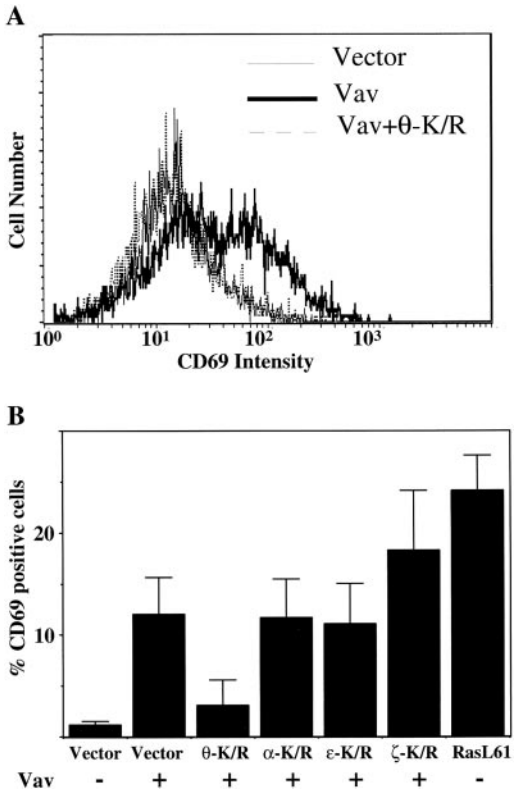

Figure 3. Vav Upregulates CD69 Expression via a Selective, PKCθ-Dependent Pathway

Jurkat-TAg cells were transfected with empty vector, Vav, or Vav plus the indicated DN PKC mutants (5 μg each) in the presence of pEF-GFP. CD69 expression was determined 2 days later by FACScan analysis of GFP-gated cells. The results are displayed as histograms (A) or as average percentage of CD69-positive cells ± SE in three separate experiments (B). As a positive control, one group of cells was transfected with CA-Ras (RasL61) to induce CD69 expression (B).

Specific Inhibition of Vav-Mediated CD69 Upregulation by DN PKCθ

We recently found that transient overexpression of either Vav (Figure 3A) or constitutively active PKCθ (data not shown) upregulates the expression of the activation antigen CD69 in Jurkat T cells. The biological relevance of this novel finding is indicated by the report that $Vav^{-/-}$ thymocytes display a defect in CD69 expression following TCR/CD3 stimulation (Fischer et al., 1995) and, furthermore, by our finding that the Vav-induced CD69 upregulation was dependent on functional Ras (Villalba et al., unpublished data), similar to the TCR/CD3-induced expression of CD69 (D'Ambrosio et al., 1994). We determined whether PKCθ is also required for Vav-mediated CD69 upregulation by assessing the effects of DN PKCθ. Coexpression of θ-K/R inhibited the effect of Vav by ~90%. In contrast, DN mutants of other PKC isoforms, which were properly overexpressed in the cells (data not shown), failed to cause a significant inhibition of Vav-induced CD69 expression (Figure 3). As a positive control (D'Ambrosio et al., 1994), a constitutively active Ras mutant (RasL61) also upregulated the expression of CD69.

Vav-Induced Actin Cytoskeleton Reorganization Is Independent of PKCθ

Vav is required for TCR clustering, reflecting its ability to promote Rac-dependent reorganization of the actin cytoskeleton (Fischer et al., 1998; Holsinger et al., 1998; Kong et al., 1998; Penninger and Crabtree, 1999). As shown in Figure 4A, transient Vav overexpression increased the F-actin content and its accumulation in the submembranous area, which was similar to the effect induced by anti-CD3 stimulation. This effect was sensitive to cytochalasin D, which disrupts the actin cytoskeleton.

Next, we determined whether Vav-induced actin polymerization was dependent on PKCθ by quantitating the binding of phalloidin to F-actin. Transient Vav overexpression increased the amount of F-actin in either unstimulated or anti-CD3-stimulated T cells (Figure 4B). In contrast to the selective requirement of PKCθ for Vav-induced transcriptional activation (Figure 1), JNK activation (Figure 2), or CD69 upregulation (Figure 3), DN PKCθ consistently failed to significantly reduce the Vav-mediated actin polymerization in unstimulated or anti-CD3-activated cells (Figure 4B, left panel). Similarly, transient overexpression of θ-K/R alone also did not interfere with the OKT3-stimulated F-actin accumulation. Consistent with these findings, the selective PKCθ inhibitor (rottlerin; Figure 1G) did not significantly inhibit Vav- or anti-CD3-induced actin polymerization (data not shown). These results indicate that Vav participates in at least

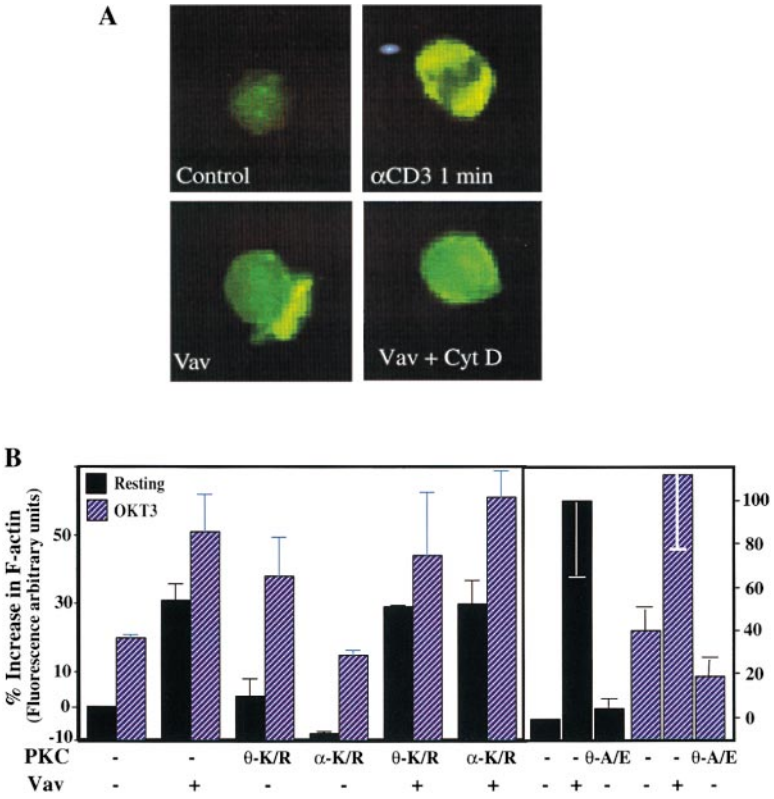

Figure 4. Vav Regulation of Actin Polymerization Is PKCθ Independent

(A) Empty vector (pEF)-transfected Jurkat T cells were left unstimulated or activated with OKT3 for 1 min (left and right upper panels, respectively). Another group of cells was transfected for 48 hr with Vav, and a portion of these cells was treated with cytochalasin D (10 μg/ml). The cells were fixed and permeabilized, and F-actin was visualized by TRITC-phalloidin staining and immunofluorescence microscopy.

(B) Jurkat-TAg cells (10×10^6) were cotransfected with the indicated combinations of Vav (5 μg) DN (K/R) or CA (A/E) PKCθ or α mutants (10 μg each) plus pEF-GFP (5 μg). Half of the cells in each group were stimulated with OKT3 (1 μg/ml) for the final 10 min of culture. After 2 days, the cells were fixed and analyzed for F-actin content as in (A). The results are displayed as percentage increase in mean fluorescence intensity relative to empty vector-transfected, unstimulated cells, and represent the mean ± SD of three (left panel) or two (right panel) experiments.

two distinct signaling pathways, i.e., a PKCθ-dependent pathway that upregulates CD69 and activates JNK and the IL-2 gene and a PKCθ-independent pathway that mediates actin polymerization and, therefore, is most likely involved in TCR/CD3 capping (Penninger and Crabtree, 1999). The independence of the latter pathway on PKCθ is further supported by the finding that, unlike Vav, transient overexpression of constitutively active PKCθ failed to induce significant F-actin accumulation (Figure 4B, right panel).

Vav Promotes the Selective Membrane Translocation of PKCθ

A recent study reported an association between PKCθ and Vav (Kong et al., 1998). However, numerous coimmunoprecipitation experiments using anti-Vav or -PKCθ antibodies failed to demonstrate detectable association between these two proteins either before or after stimulation with anti-CD3 and/or anti-CD28 antibodies (Figure 5A), even when they were overexpressed by transient transfection. Under the same conditions, the association between Vav and the adapter protein SLP-76 (Wu et al., 1996) and its upregulation by T cell activation could be readily demonstrated (Figure 5A). It is, therefore, possible that the reported Vav-PKCθ association (Kong et al., 1998) is a unique feature of immature T cells.

PKCθ activation is associated with its membrane and cytoskeletal translocation (Meller et al., 1996) as well as with its colocalization with the TCR to the contact site between antigen-specific T cells and APCs (Monks et al., 1997). Consistent with this, costimulation of Jurkat T cells with anti-CD3 plus anti-CD28 antibodies induced a time-dependent translocation of PKCθ from the cytosol to the membrane and detergent-insoluble (cytoskeleton) subcellular fractions, which peaked at 10 min (Figure 5B). We ascertained whether transient Vav overexpression could promote a similar translocation of PKC. Jurkat cells were transfected with Vav, and the distribution of endogenous PKCθ, α, ε, and ζ among the cytosol, membrane, and detergent-insoluble fractions was determined by immunoblotting. The results (Figure 5C) show an increase in the membrane and cytoskeletal fractions of PKCθ relative to its cytosolic content, but not of the other PKC isoforms, in the Vav-transfected cells. This selective translocation of PKCθ is evident after quantitation of the PKC signals (Figure 5D). In contrast to the selective effect of Vav, PMA treatment induced cytosol-to-membrane translocation of PKCθ, α, and ε but had no effect on PKCζ, which is unresponsive to PMA (Ohno et al., 1989). The effect of Vav on PKCθ localization probably represents an underestimate since only a fraction of the cell population expressed the transfected Vav vector.

Next, we cotransfected Jurkat cells with an epitope-tagged PKCθ expression vector plus wild-type or DN (ΔPH) Vav and analyzed the expression of the transfected PKCθ in different subcellular fractions by immunoblotting with an anti-Xpress tag mAb (Figure 5E). The results clearly indicate that anti-CD3 stimulation as well as Vav coexpression caused a similar redistribution of the cotransfected PKCθ from the cytosol to the membrane and cytoskeleton fractions. More importantly, the basal expression of PKCθ in the membrane and cytoskeleton and, in particular, its OKT3-induced translocation to the same fractions was severely reduced by coexpressing a DN Vav mutant (second panels from bottom). In addition, the effect of wild-type Vav was reversed by coexpressing a DN Rac mutant (bottom panels). We confirmed these effects of Vav by using

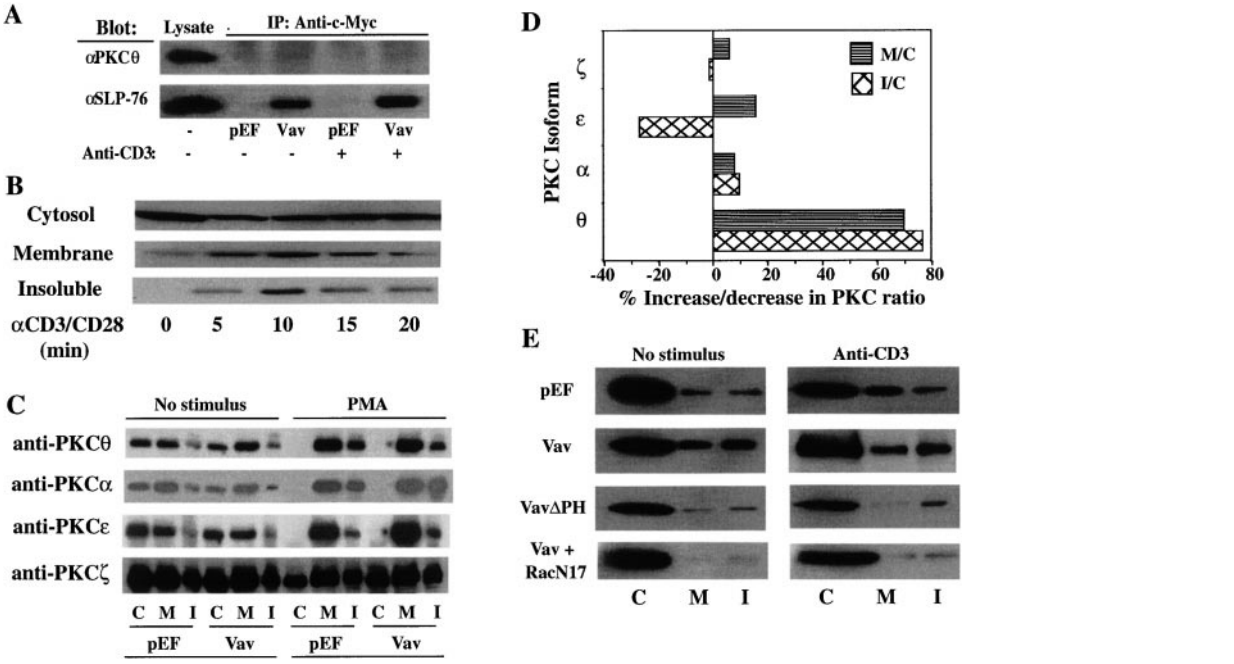

Figure 5. Vav Induces Membrane and Cytoskeleton Translocation of PKCθ

(A) Jurkat-TAg cells were transfected with empty vector (pEF) or pEF-c-Myc-Vav (10 μg) and left unstimulated or activated with OKT3 for the final 2 min of a 48 hr culture. The cells were lysed, and Vav was immunoprecipitated with an anti-c-Myc mAb. The immunoprecipitates, or a sample of the cell lysate, were analyzed by immunoblotting with anti-PKCθ or -SLP-76 antibodies. Similar results were obtained in several experiments.

(B) Jurkat cells were stimulated with anti-CD3 plus anti-CD28 antibodies for the indicated times. Cytosol, membrane, and detergent-insoluble fractions were prepared, resolved by SDS-PAGE, and the expression of PKCθ in each fraction was determined by immunoblotting with a specific antibody. These results are representative of five similar experiments.

(C) Vav induces selective translocation of endogenous PKCθ to the membrane and cytoskeleton. Jurkat-TAg cells (20×10^6) were transfected with empty vector (pEF) or Vav (5 μg each) and lysed after 40 hr. Cytosol (C), membrane (M), and detergent-insoluble (I) cell fractions were prepared, resolved by SDS-PAGE, and expression of the indicated endogenous PKC isoforms in each fraction was analyzed by immunoblotting with the corresponding isoform-specific antibodies (left panels). As a positive control for PKC translocation, a portion of the cells in each transfection group was stimulated with PMA (100 ng/ml) for the final 10 min of culture (right panels). The same protein amounts from each fraction were loaded in each lane. Similar findings were obtained in a separate experiment.

(D) PKC signals from the unstimulated cells in (C) were quantitated by scanning densitometry, normalized to an equal cell number in each fraction, and the membrane-to-cytosol (M/C) or cytoskeleton-to-cytosol (I/C) PKC ratios were calculated. The results are displayed as percentage of increase/decrease of the PKC ratio in the Vav-transfected cells relative to pEF-transfected cells.

(E) PKCθ translocation is regulated by Vav. Jurkat-TAg cells (20×10^6) were transfected with the indicated combinations of wild-type Vav (5 μg), DN-Vav (ΔPH), and/or DN-Rac (RacN17) (10 μg each) together with an Xpress-tagged PKCθ plasmid (5 μg). A portion of the cells in each group was stimulated with OKT3 (1 μg/ml) for the final 10 min of culture. Cells were lysed and fractionated as in (C), and expression of the transfected PKCθ in each fraction was determined by anti-Xpress immunoblotting. These results are representative of three similar experiments.

confocal microscopy to follow the localization of a transfected recombinant protein consisting of the regulatory domain of PKCθ fused to green fluorescent protein (GFP). Similar constructs of other PKCs were found to mimic the behavior of native PKC and its stimulus-dependent response (Oancea and Meyer, 1998). F-actin distribution was followed in parallel by phalloidin staining. As reported (Meller et al., 1996), a fraction of PKCθ-GFP localized to the membrane in unstimulated Jurkat cells (Figure 6, top right panel). PMA stimulation (middle right panel) as well as Vav overexpression (bottom right panel) induced increased translocation and a more granular appearance of PKCθ to the membrane and submembranous area, and this coincided with the redistribution of F-actin in the same cells (left panels). A similar result was obtained when heterologous 293 cells were cotransfected with Vav and the PKCθ-GFP plasmids (data not shown). Consistent with this effect of Vav, transient Vav overexpression enhanced the redistribution of PKCθ into clustered membrane lipid rafts (K. Bi and A. A., unpublished data). Taken together, the results in Figures 5 and 6 indicate that Vav-dependent signals, which most likely reflect its regulation of the actin cytoskeleton (Figure 4), are important for the translocation of PKCθ from the cytosol into compartments where it can potentially interact with the TCR signaling machinery (Monks et al., 1997) and mediate its biological activities.

Vav Activates PKCθ and Is Involved in Receptor-Mediated PKCθ Activation

In order to determine whether the Vav-induced translocation of PKCθ to the membrane and cytoskeleton is associated with its enzymatic activation, we performed immune complex kinase assays of PKCθ isolated from

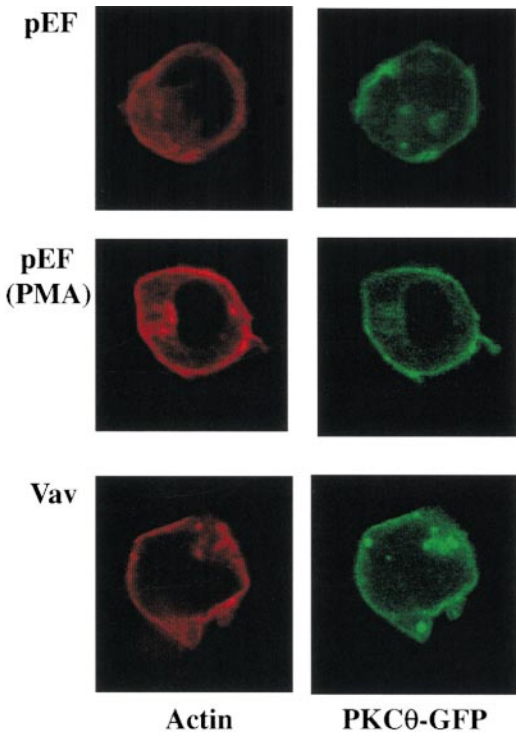

Figure 6. Vav Induces Membrane Translocation of a PKCθ-GFP Fusion Protein

Jurkat-TAg cells were transfected with empty vector (pEF) or Vav (5 μg each) together with 5 μg of a PKCθ regulatory domain-GFP expression plasmid. After 2 days, GFP localization was analyzed by confocal imaging. Some cells were stimulated for the final 10 min of culture with PMA (100 ng/ml) as a positive control for PKCθ translocation. F-actin localization in the same cells was revealed by phalloidin staining. These images are representative of 80% of the pEF-transfected cells (43/50 cells observed), 95% of the PMA-stimulated cells (26/27 cells observed), and 75% of the Vav-transfected cells (32/41 cells observed).

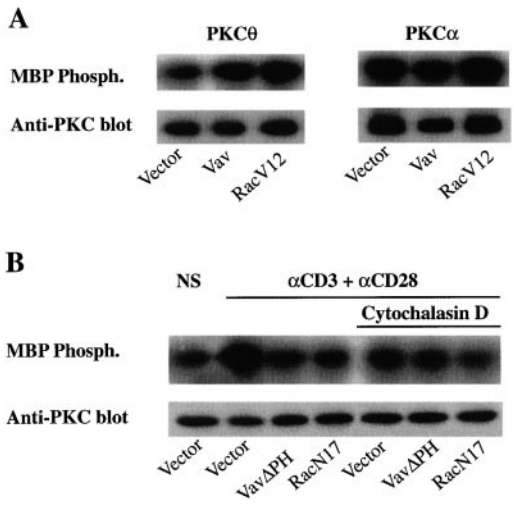

Figure 7. A Vav/Rac/Cytoskeleton Pathway Mediates Receptor-Stimulated PKCθ Activation

(A) Jurkat-TAg cells were transfected with 5 μg empty vector, Vav, or CA-Rac (RacV12). Endogenous PKCθ or PKCα was immunoprecipitated after 40 hr, and enzymatic activity was determined by an in vitro kinase reaction using MBP as a substrate (top panels). The kinase reactions were performed in the absence of lipid cofactors or PMA to reflect the in situ activity of the kinases (Monks et al., 1997). Aliquots of the immunoprecipitates were immunoblotted with the corresponding PKC-specific antibodies to confirm the similar expression levels of PKCθ or α in different groups (bottom panels). (B) Jurkat-TAg cells were transfected with the indicated plasmids and were either left unstimulated or stimulated with anti-CD3 plus anti-CD28 antibodies for the final 10 min of culture. Some groups were pretreated with cytochalasin D (10 μg/ml) as indicated. PKCθ activity (top panel) or expression levels (bottom panel) were determined as in (A). Similar results to those in (A) and (B) were obtained in two additional experiments.

empty vector-transfected cells or from cells cotransfected with Vav or a CA Rac (RacV12) mutant. The kinase assays were performed in the absence of the activating lipid cofactors in order to assess the in situ activity of PKC (Monks et al., 1997). Vav as well as RacV12 induced activation of PKCθ, and this effect was selective since activation of PKCα was not detected (Figure 7A).

Finally, we analyzed the effects of DN Vav (VavΔPH) or Rac (RacN17) mutants on the anti-CD3/CD28-stimulated activation of PKCθ. This stimulation induced a readily detectable activation of PKCθ (Figure 7B; lane 2 versus lane 1). However, PKCθ activation was blocked when the cells were cotransfected with the DN Vav (lane 3) or Rac (lane 4) mutants. For comparison, cytochalasin D pretreatment also blocked the ability of the antibodies to induce PKCθ activation (lane 5) or actin polymerization (Figure 4A). Nearly equal amounts of PKCθ were expressed in all groups (Figure 7B, bottom panel).

Discussion

Productive T cell activation requires the proper assembly and formation of the T cell SMAC (Monks et al., 1998) or synapse (Grakoui et al., 1999) in the contact region with APCs, a process that depends on reorganization of the actin cytoskeleton (Penninger and Crabtree, 1999) and on the selective accumulation of the TCR/CD3 complex and associated signaling molecules within membrane lipid rafts (Xavier and Seed, 1999). Our findings reveal a novel role for Vav in the recruitment of PKCθ to the relevant membrane/cytoskeletal compartments, where it needs to localize in order to become activated and promote TCR-initiated proliferation (Monks et al., 1997, 1998). The relevance of these results to physiological events associated with TCR/CD3-mediated T cell activation is established by the following: the ability of DN Vav or PKCθ mutants to inhibit CD3 or CD3/CD28-mediated transcriptional activation (Figure 1) or JNK activation (Werlen et al., 1998; Villalba et al., unpublished data); the inhibition of antigen- and/or Vav-induced NFAT activity by a selective PKCθ inhibitor (Figure 1G); the translocation and activation of PKCθ induced by physiological CD3/CD28 stimulation (Figure 5A), which is mimicked by Vav (Figures 5B–5D and 6); and the inhibition of receptor-induced PKCθ translocation (Figure 5D) or activation (Figure 7B) by DN Vav or Rac and by cytochalasin D. Taken together, all of these findings and, in particular, the similarities between the actions of Vav and PKCθ at the level of transcriptional stimulation, JNK activation, and CD69 upregulation suggest

that Vav and PKCθ are components of the same signaling pathway. Moreover, the findings that the above functional effects induced by Vav are blocked by DN PKCθ but not vice versa indicate that PKCθ can be functionally placed downstream of Vav in this pathway.

Despite these similarities, there are some notable differences between Vav and PKCθ. Thus, PKCθ is considerably more efficient than Vav in stimulating the IL-2 gene promoter and particularly AP-1 in unstimulated cells; conversely, Vav is a more potent inducer of NFAT activity. We attribute these differences to the fact that PKCθ is an effective activator of the Ras/JNK/AP-1 pathway (Baier-Bitterlich et al., 1996; Werlen et al., 1998; Ghaffari-Tabrizi et al., 1999) but, most likely, does not have a role in activating the Ca^{2+}/Cn pathway; in contrast, Vav is a weak inducer of the Ras/JNK pathway (Villalba et al., unpublished data), but it plays an important role in activating the Ca^{2+}/Cn pathway (Fischer et al., 1998; Holsinger et al., 1998; Costello et al., 1999), which is essential for NFAT induction (Rao et al., 1997; Crabtree, 1999). Based on these differences, we favor the view that Vav and PKCθ function in overlapping but not identical pathways. One pathway, which is shared by both signaling proteins, mediates transcription factor and JNK activation as well as CD69 upregulation. However, each of these proteins uniquely regulates other nonoverlapping signaling events (Collins et al., 1997; Meller et al., 1998).

Despite being able to block transcriptional activation, JNK stimulation, and CD69 upregulation (Figures 1–3), DN PKCθ has no detectable effect on the Vav- or anti-CD3-induced F-actin accumulation (Figure 4B). The most plausible model to explain this finding is as follows: first, TCR/CD28 stimulation activates tyrosine kinases, which, in turn, activate Vav via its tyrosine phosphorylation in a process that is not strictly dependent on actin cytoskeleton reorganization. This is consistent with the findings that early tyrosine phosphorylation events are intact in cytochalasin D–treated normal T cells or in Vav-deficient T cells (Fischer et al., 1998; Holsinger et al., 1998). Second, activated Vav induces actin polymerization and TCR capping via Rac/Cdc42, and this process does not require functional PKCθ. However, these Vav-induced events are essential for the translocation of PKCθ and its colocalization with the TCR in the T cell SMAC (Monks et al., 1997, 1998) as well as for its enzymatic activation. Direct or indirect association of PKCθ with a cytoskeletal protein could represent one mechanism through which Vav may promote PKCθ translocation and activation. Finally, the Vav/Rac-induced membrane recruitment of PKCθ induces its activation and, consequently, activation of JNK and the IL-2 gene as well as upregulation of CD69. The dependence of this pathway on intact PKCθ function reflects the essential role of a Vav/Rac pathway in inducing PKCθ membrane localization and activation. It would be interesting to determine whether this represents an alternative PKCθ activation mechanism that is independent of PLCγ1 activation and diacylglycerol production.

According to this model, Vav independently regulates different T cell functions, as would be expected from its domain structure and multifunctional nature (Collins et al., 1997). This notion is generally consistent with the recently emerging view that Vav independently regulates different signaling pathways, including growth and cytoskeleton reorganization pathways (Cantrell, 1998; Fischer et al., 1998). It is also consistent with the findings that distinct functions of the Vav effectors, Rac or Cdc42, i.e., their ability to induce reorganization of the actin cytoskeleton and JNK activation versus the activation of PAK, can be dissociated by mutations in their effector domains (Lamarche et al., 1996).

The major novel aspect of our study is the functional linkage of PKCθ to Vav and Rac in the T cell growth regulatory pathway and the apparent absolute dependence of Vav on intact PKCθ function in this pathway. This link between Vav and PKCθ provides a mechanistic basis for the implied connection between these two proteins suggested recently by Penninger and Crabtree (1999). However, since we failed to confirm the reported (Kong et al., 1998) physical association between Vav and PKCθ (Figure 5A), it is likely that, at least in our system, PKCθ is functionally but not physically linked to Vav. It is possible that the reported association between Vav and PKCθ (Kong et al., 1998) reflects their localization to the same membrane microdomains and/or cytoskeletal structures, rather than a direct physical interaction.

The model proposed herein provides a common mechanistic basis for several recent findings concerning the assembly and organization of the T cell synapse (Grakoui et al., 1999) or SMAC (Monks et al., 1998): first, the selective antigen-induced activation and localization of PKCθ to the contact site between antigen-specific T cells and APCs (Monks et al., 1997); second, the selective role of PKCθ in JNK, AP-1, and IL-2 induction (Baier-Bitterlich et al., 1996; Werlen et al., 1998; Ghaffari-Tabrizi et al., 1999); third, the importance of Vav in reorganizing the T cell actin cytoskeleton and TCR cap (Fischer et al., 1998; Holsinger et al., 1998); fourth, its accumulation in the same lipid rafts where the TCR complex and various signaling elements, including (presumably) PKCθ (Monks et al., 1997), are found (Xavier and Seed, 1999); and, finally, the ability of Vav to independently regulate actin polymerization and growth signals (our findings herein; Miranti et al., 1998). The availability of Vav-deficient mice and the anticipated generation of PKCθ-deficient mouse lines will now make it possible to test several experimental predictions that can be formulated based on the model emerging from our findings.

Experimental Procedures

Antibodies and Reagents

Polyclonal rabbit antibodies against PKCθ and JNK and the anti-Myc mAb (9E10) were obtained from Santa Cruz Biotechnology. PKCθ-, α-, ε-, or ζ-specific mAbs were obtained from Transduction Laboratories. The anti-CD3 mAb (OKT3) was purified as previously described (Villalba et al., 1999a). The anti-human CD28 and phycoerythrin (PE)-conjugated anti-human CD69 mAbs were from PharMingen. The anti-HA (clone 12CA5) and -Xpress tag mAbs were obtained from Roche Molecular Biochemicals and Invitrogen, respectively. The anti-phospho-c-Jun polyclonal antibody was from New England Biolabs, and the anti-human Vav mAb was from Upstate Biotechnology. Donkey anti-rabbit or sheep anti-mouse IgG antibodies were obtained from Amersham. Rottlerin and Gö6976 were purchased from Calbiochem. All other reagents were obtained from Sigma.

Plasmids

The cDNAs encoding human wild-type PKCθ and α, the corresponding constitutively active (A/E) mutants, and the DN (kinase-inactive; K/R) mutants of these PKCs as well as rat PKCε and mouse PKCζ in the eukaryotic expression vector pEF*neo* have been described (Villalba et al., 1999). To generate an epitope-tagged version of PKCθ, the PKCθ plasmids were subcloned into the BamHI and XbaI sites, respectively, of the pEF4/His-C mammalian expression vector (Invitrogen) by standard techniques. This vector encodes in-frame His_6 and Xpress tags upstream of the insert. The construction of c-Myc-tagged Vav expression plasmids in the pEF vector was described (Villalba et al., unpublished data). Briefly, the PH domain of wild-type Vav cDNA (Deckert et al., 1996) was deleted (ΔPH) by digesting the corresponding cDNA with NgoMI and BspEI, thereby excising a fragment encoding residues 403–507 of Vav. An HA-tagged, constitutively active calcineurin A mutant (CA-Cn), a CA Ras (RasL61) mutant, CA (RacV12) or DN (RacN17) Rac mutants (all in the pSRα vector), and HA-tagged JNK1 in pCDNA3 were obtained from Dr. M. Karin and have been described (Werlen et al., 1998; Villalba et al., unpublished data). An expression vector encoding the regulatory domain of PKCθ fused to the N terminus of GFP was a generous gift from Dr. J. Neefjes and E. Reits (The Netherlands Cancer Institute, Amsterdam). This plasmid was generated by PCR-mediated amplification of the PKCθ regulatory domain and its ligation into the pEGFP-N1 vector (Clontech). A GFP expression plasmid in the pEF vector was obtained from Dr. G. Baier (University of Innsbruck). As control for transfection efficiencies, a β-galactosidase (β-gal) expression plasmid in the pEF vector was used.

Cell Culture and Transfection

Simian virus 40 large T antigen–transfected human leukemic Jurkat T (Jurkat-TAg) cells were grown in RPMI 1640 medium (Life Technologies), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1× MEM nonessential amino acid solution (Life Technologies), and 100 μ/ml each of penicillin G and streptomycin. CH7C17 is a Jurkat variant that stably expresses an HA peptide (residues 307–319) specific TCR (Hewitt et al., 1992). The EBV-transformed human B lymphoblastoid cell line LG-2 was used as a source of mitomycin C–treated APCs. COS cells and simian virus 40 large T antigen–transfected 293 (293 T) cells were grown in DMEM (Life Technologies) supplemented with 2 mM glutamine, 10% FBS, 1 mM sodium pyruvate, and the same antibiotics. Cells in a logarithmic growth phase were transfected with the indicated amounts of plasmid DNAs by electroporation as described previously (Villalba et al., 1999a).

Luciferase and β-Galactosidase Assays

Transfected Jurkat-TAg cells were harvested after 2 days, washed twice with PBS, lysed, and luciferase or β-galactosidase activities in cell extracts were determined as described (Villalba et al., 1999a, unpublished data).

Immunoprecipitation and Immunoblotting

Cells were lysed in 1 ml lysis buffer (20 mM Tris–HCl [pH 7.5] 150 mM NaCl, 5 mM EDTA, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4 plus 1% Nonidet P-40 [NP-40] and 10 μg/ml each of aprotinin and leupeptin) for 10 min on ice. After centrifugation (16,000 × g, 10 min at 4°C), the supernatants were incubated with optimal concentrations of antibodies for 1 hr at 4°C, followed by 30 μl of protein A/G-plus-agarose (Santa Cruz Biotechnology) and overnight incubation at 4°C. Samples were washed four times in lysis buffer, precipitates were dissolved in Laemmli buffer and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Electrophoresed samples were processed for Western blot analysis as previously described (Villalba et al., 1999).

Kinase Assays

For PKC assays, 20 × 10^6 Jurkat or 2 × 10^6 293 cells were lysed, and PKC was immunoprecipitated as described above. Immunoprecipitates were washed two times with lysis buffer, two times with PKC kinase buffer (20 mM HEPES, 10 mM $MgCl_2$, and 0.1 mM EGTA (PKCθ) or 10 mM $MgCl_2$ and 0.1 mM $CaCl_2$ [PKCα]), and resuspended in 20 μl of the corresponding kinase buffer plus 10 μCi [γ-^{32}P]ATP, 100 μM ATP, and 2 μg of myelin basic protein (MBP) as substrate. Samples were incubated for 20 min at 30°C with gentle shaking. The reaction was stopped by adding 20 μl of 2× Laemmli buffer. Samples were incubated at 100°C for 5 min and subjected to SDS-PAGE and autoradiography. [γ-^{32}]ATP incorporation was determined using a STORM 860 PhosphorImager (Molecular Dynamics).

Transfected JNK1 was immunoprecipitated with an anti-HA mAb as described above, and its activity was assayed in 20 μl of JNK kinase buffer (25 mM HEPES [pH 7.5], 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 1 mM DTT, and 0.1 mM Na_3VO_4) for 20 min at 30°C with gentle shaking, using 2 μg of GST-c-Jun as a substrate. Reactions were stopped and processed as described above.

CD69 Expression

Cells were transfected with the indicated vectors plus a GFP expression plasmid and harvested 2 days later. The cells were washed in RPMI-1640 medium and suspended in 500 μl of RPMI-1640 plus 20 μl of PE-conjugated anti-human CD69 mAb for 15 min. CD69 expression was analyzed by flow cytometry (FACScan, Becton and Dickinson) after gating on GFP-positive cells.

Actin Polymerization

Jurkat-TAg cells (10 × 10^6 per condition) were transfected with the indicated Vav or PKCθ vectors plus 5 μg pEF-GFP and cultured for 36 hr in the absence or presence of cytochalasin D (10 μM). The cells were collected, washed, resuspended in RPMI plus 10% FBS, and prewarmed for 15 min at 37°C. Some groups were stimulated with an anti-CD3 mAb (OKT3; 5 μg/ml) for the indicated times. Reactions were stopped by adding 1 ml of 3.7% paraformaldehyde in PBS and incubating for 15 min at room temperature, followed by blocking with PBS/1% BSA/0.1% NaN_3 under the same conditions. Following centrifugation (2000 rpm, 3 min), the pellet was carefully resuspended in PBS/1% BSA/0.1% NaN_3/0.05% saponin and incubated for 30 min at 4°C in order to permeabilize the cells. The cells were centrifuged again, resuspended in the same solution plus 100 ng/ml TRITC-conjugated phalloidin (Sigma), and incubated for 30–60 min at 4°C in the dark. Following three washes with cold PBS/1% BSA/0.1% NaN_3, GFP-gated cells were analyzed by flow cytometry.

Immunofluorescence Microscopy

Jurkat-TAg cells were transfected with the corresponding plasmids, harvested 2 days later, washed, fixed, and permeabilized as described above. The cells were incubated overnight at 4°C with 1 μg/ml of an anti-PKCθ antibody in 50 μl of PBS/1% BSA, washed three times in the same medium, and incubated for 30 min at room temperature in the dark with FITC-conjugated goat anti-rabbit IgG antibody (1:100 dilution; Cappel) to visualize PKCθ. The cells were stained in parallel with TRITC-phalloidin as described above. After three washes with ice-cold PBS/1% BSA, the cells were mounted on coverglasses using a SlowFade kit (Molecular Probes) and analyzed by conventional fluorescence microscopy or by confocal microscopy (MRC 1024S; Bio-Rad).

Subcellular Fractionation

Cells were resuspended in ice-cold hypotonic buffer (42 mM KCl, 10 mM HEPES [pH 7.4], 5 mM $MgCl_2$, 10 μg/ml each aprotinin and leupeptin) and incubated on ice for 15 min. Cells were transferred to a 1 ml syringe and sheared by being passed five times through a 30 gauge needle. The lysates were centrifuged at 200 × g for 10 min to remove nuclei and cell debris, and 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 (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% NP-40, and 10 μg/ml each aprotinin and leupeptin), 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 a final concentration of 1× Laemmli buffer and separated by SDS-PAGE.

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References

Baier, G., Telford, D., Giampa, L., Coggeshall, K.M., Baier-Bitterlich, G., Isakov, N., and Altman, A. (1993). Molecular cloning and characterization of PKCθ, a human novel member of the protein kinase C (PKC) gene family expressed predominantly in hematopoietic cells. J. Biol. Chem. *268*, 4997–5004.

Baier-Bitterlich, G., Überall, F., Bauer, B., Fresser, F., Wachter, H., Grünicke, H., Utermann, G., Altman, A., and Baier, G. (1996). PKCθ isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. Mol. Cell Biol. *16*, 1842–1850.

Cantrell, D. (1998). Lymphocyte signaling: a coordinating role for Vav? Curr. Biol. *8*, R535–R538.

Chen, C.Y., Forman, L.W., and Faller, D.V. (1996). Calcium-dependent immediate-early gene induction in lymphocytes is negatively regulated by p21Ha-ras. Mol. Cell. Biol. *16*, 6582–6592.

Collins, T., Deckert, M., and Altman, A. (1997). Views on Vav. Immunol. Today *18*, 221–225.

Costello, P.S., Walters, A.E., Mee, P.J., Turner, M., Reynolds, L.F., Prisco, A., Sarner, N., Zamoyska, R., and Tybulewicz, V.L.J. (1999). The Rho-family GTP exchange factor Vav is a critical transducer of T cell receptor signals to the calcium, ERK, and NF-κB pathways. Proc. Natl. Acad. Sci. USA *96*, 3035–3040.

Coudronniere, N., Villalba, M., Englund, N., and Altman, A. (2000). NF-κB activation induced by CD28 costimulation is mediated by PKCθ. Proc. Natl. Acad. Sci. USA, in press.

Crabtree, G.R. (1999). Genetic signals and specific outcomes: signaling through Ca^{2+}, calcineurin and NF-AT. Cell *96*, 611–614.

Crabtree, G.R., and Clipstone, N.A. (1994). Signal transmission between the plasma membrane, and nucleus of T lymphocytes. Annu. Rev. Biochem. *63*, 1045–1083.

D'Ambrosio, D., Cantrell, D.A., Frati, L., Santoni, A., and Testi, R. (1994). Involvement of $p21^{ras}$ in T cell CD69 expression. Eur. J. Immunol. *24*, 616–620.

Deckert, M., Tartare Deckert, S., Couture, C., Mustelin, T., and Altman, A. (1996). Functional and physical interactions of Syk family kinases with the Vav proto-oncogene product. Immunity *5*, 591–604.

Fischer, K.D., Zmuldzinas, A., Gardner, S., Barbacid, M., Bernstein, A., and Guidos, C. (1995). Defective T-cell receptor signaling and positive selection of Vav-deficient CD4+ CD8+ thymocytes. Nature *374*, 474–477.

Fischer, K.D., Kong, Y.Y., Nishina, H., Tedford, K., Marengère, L.E., Kozieradzki, I., Sasaki, T., Starr, M., Chan, G., Gardener, S., et al. (1998). Vav is a regulator of cytoskeletal reorganization mediated by the T-cell receptor. Curr. Biol. *8*, 554–562.

Ghaffari-Tabrizi, N., Bauer, B., Altman, A., Utermann, G., Uberall, F., and Baier, G. (1999). Protein kinase Cθ, a selective upstream regulator of JNK/SAPK and IL-2 promoter activation in Jurkat T-cells. Eur. J. Immunol. *29*, 132–142.

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.

Hewitt, C.R.A., Lamb, J.R., Hayball, J., Hill, M., Owen, M.J., and O'Hehir, R.E. (1992). Major histocompatibility complex independent clonal T cell anergy by direct interaction of *Staphylococcus aureus* enterotoxin B with the T cell antigen receptor. J. Exp. Med. *175*, 1493–1499.

Holsinger, L.J., Graef, I.A., Swat, W., Chi, T., Bautista, D.M., Davidson, L., Lewis, R.S., Alt, F.W., and Crabtree, G.R. (1998). Defects in actin-cap formation in Vav-deficient mice implicate an actin requirement for lymphocyte signal transduction. Curr. Biol. *8*, 563–572.

Jain, J., Loh, C., and Rao, A. (1995). Transcriptional regulation of the IL-2 gene. Curr. Opin. Immunol. *7*, 333–342.

Kong, Y.Y., Fischer, K.D., Bachmann, M.F., Mariathasan, S., Kozieradzki, I., Nghiem, M.P., Bouchard, D., Bernstein, A., Ohashi, P.S., and Penninger, J.M. (1998). Vav regulates peptide-specific apoptosis in thymocytes. J. Exp. Med. *188*, 2099–2111.

Lamarche, N., Tapon, N., Stowers, L., Burbelo, P.D., Aspenstrom, P., Bridges, T., Changt, J., and Hall, A. (1996). Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of $p65^{PAK}$ and the JNK/SAPK MAP kinase cascade. Cell *87*, 519–552.

Miranti, C.K., Leng, L., Maschberger, P., Brugge, J.S., and Shattil, S.J. (1998). Identification of a novel integrin signaling pathway involving the kinase Syk and the guanine nucleotide exchange factor Vav1. Curr. Biol. *8*, 1289–1299.

Meller, N., Liu, Y.C., Collins, T.L., Bonnefoy-Berard, N., Baier, G., Isakov, N., and Altman, A. (1996). Direct interaction between protein kinase C θ (PKC-θ) and 14-3- 3τ in T cells: 14-3-3 overexpression results in inhibition of PKCθ translocation and function. Mol. Cell. Biol. *16*, 5782–5791.

Meller, N., Altman, A., and Isakov, N. (1998). New perspectives on PKCθ, a member of the novel subfamily of protein kinase C. Stem Cells *16*, 178–192.

Monks, C.R.F., Kupfer, H., Tamir, I., Barlow, A., and Kupfer, A. (1997). Selective modulation of protein kinase C-θ 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 clusters in T cells. Nature *395*, 82–86.

Oancea, E., and Meyer, T. (1998). Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. Cell *95*, 307–318.

Ohno, Y., Fuji, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1989). Protein kinase C ζ subspecies from rat brain: its structure, expression, and properties. Proc. Natl. Acad. Sci. USA *86*, 3099–3103.

Penninger, J.M., and Crabtree, G.R. (1999). The actin cytoskeleton and lymphocyte activation. Cell *96*, 9–12.

Rao, A., Luo, C., and Hogan, P.G. (1997). Transcription factors of the NFAT family: regulation and function. Annu. Rev. Immunol. *15*, 707–747.

Romero, F., and Fischer, S. (1996). Structure and function of vav. Cell Signal. *8*, 545–553.

Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben Neriah, Y. (1994). JNK is involved in signal integration during costimulation of T lymphocytes. Cell *77*, 727–736.

Turner, M., Mee, P.J., Walters, A.E., Quinn, M.E., Mellor, A.L., Zamoyska, R., and Tybulewicz, V.L.J. (1997). A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. Immunity *7*, 451–460.

Villalba, M., Kasibhatla, S., Genestier, L., Mahboubi, A., Green, D.R., and Altman, A. (1999). PKCθ is a necessary component, and cooperates with calcineurin, to induce FasL expression during activation-induced T cell death. J. Immunol. *163*, 5813–5819.

Weiss, A., and Littman, D.R. (1994). Signal transduction by lymphocyte antigen receptors. Cell *76*, 263–274.

Werlen, G., Jacinto, E., Xia, Y., and Karin, M. (1998). Calcineurin preferentially synergizes with PKC-θ to activate JNK and IL-2 promoter in T lymphocytes. EMBO J. *17*, 3101–3111.

Wu, J., Katzav, S., and Weiss, A. (1995). A functional T-cell receptor signaling pathway is required for $p95^{vav}$ activity. Mol. Cell. Biol. *15*, 4337–4346.

Wu, J., Motto, D.G., Koretzky, G.A., and Weiss, A. (1996). Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. Immunity *4*, 593–602.

Xavier, R., and Seed, B. (1999). Membrane compartmentation and the response to antigen. Curr. Opin. Immunol. *11*, 265–269.

Zhang, R., Alt, F.W., Davidson, L., Orkin, S.H., and Swat, W. (1995). Defective signaling through the T- and B-cell antigen receptors in lymphoid cells lacking the *vav* proto-oncogene. Nature *374*, 470–473.