Membrane Translocation of Protein Kinase Cθ during T Lymphocyte Activation Requires Phospholipase C-γ-generated Diacylglycerol*

Received for publication, March 27, 2003, and in revised form, May 7, 2003
Published, JBC Papers in Press, May 8, 2003, DOI 10.1074/jbc.M303165200

Ernesto Díaz-Flores‡, Maria Siliceo‡, Carlos Martínez-A., and Isabel Mérida§

From the Department of Immunology and Oncology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Cantoblanco, E-28049 Madrid, Spain

Protein kinase Cθ (PKCθ) is the only PKC isoform recruited to the immunological synapse after T cell receptor stimulation, suggesting that its activation mechanism differs from that of the other isoforms. Previous studies have suggested that this selective PKCθ recruitment may operate via a Ca2+/inositol 1,4,5-triphosphate-dependent mechanism, independent of the classical phospholipase C/diacylglycerol pathway. Here, we demonstrate that, together with tyrosine phosphorylation of PKCθ in the regulatory domain, binding of phospholipase C-dependent diacylglycerol is required for PKCθ recruitment to the T cell synapse. In addition, we demonstrate that diacylglycerol kinase θ-dependent diacylglycerol phosphorylation provides the negative signal required for PKCθ inactivation, ensuring fine control of the T cell activation response.

During an immune response, T lymphocyte interaction with an antigen-presenting cell (APC) at the so-called immunological synapse (1) governs lymphocyte responsiveness. The molecular signaling complex formed at the cell-cell contact site triggers a cascade of biochemical events, culminating in cell proliferation and the execution of T cell effector functions (2).

An important consequence of T cell receptor (TCR) stimulation is phospholipase C-γ (PLC-γ) activation. This enzyme is recruited to the membrane by adapter proteins and is also activated directly by tyrosine phosphorylation (3, 4). The activated enzyme hydrolyzes phosphatidylinositol 4,5-diphosphate to inositol 1,4,5-triphosphate, which stimulates Ca2+ release from intracellular stores and to diacylglycerol (DAG). The importance of Ca2+ and DAG in T cell activation was demonstrated several years ago, when it was shown that a combination of Ca2+ ionophore and phorbol esters, which function as DAG analogues, could mimic TCR signals, leading to full T cell activation (5). The relevance of PLC-γ in TCR signals was further highlighted by the impaired TCR activation in PLC-γ-deficient cell lines (6). Similarly, mutations in the T cell adapter LAT, which prevents PLC-γ recruitment and activation (3), abrogate expression of several T cell activation-associated genes, including interleukin-2 (IL-2) (7). PLC-γ1-dependent Ca2+ elevation activates the phosphatase calcineurin, leading to de-phosphorylation of the transcription factor NF-AT (8). The exact role of PLC-γ-generated DAG nonetheless remains a matter of controversy. According to conventional models of TCR signaling, cell responses to increased DAG are due to protein kinase C (PKC) activation (9). The recent discovery of RasGRP, a Ras guanine exchange factor containing a DAG-binding C1-like domain, has nevertheless established a direct link between TCR-mediated PLC-γ activation and the Ras-extracellular signal-regulated kinase pathway (10).

Is well established that DAG generation is responsible for membrane translocation and activation of classical and novel PKC family members (11, 12). T lymphocytes express several classical and novel PKC isoforms, although only the novel isoform PKCθ is selectively recruited to the immunological synapse (13). Analysis of PKCθ knockout mice showed the importance of this isoform in regulating TCR-derived signals and demonstrated the requirement for PKCθ in activating the downstream elements AP-1, NF-κB, and IL-2 in T cells (14). There is thus great interest in dissecting the mechanism underlying selective PKCθ recruitment to TCR contact sites during antigen stimulation. Recent experiments suggested that PKCθ recruitment to the cell membrane is mediated by Vav/Rac activation through a PLC-γ-independent mechanism that involves cytoskeletal reorganization (15–19). Nonetheless, analysis of Vav1-deficient mice showed that during T cell activation, this exchange factor for Rac acts as an upstream regulator of PLC-γ1 (20). According to these results, PLC-mediated DAG generation would be a direct consequence of Vav-dependant cytoskeletal reorganization, making it difficult to assess the specific contribution of DAG generation among Vav-regulated signals.

Here we used several approaches to evaluate the exact contribution of PLC-γ-dependent DAG generation in the control of TCR-mediated PKCθ recruitment and activation. We used pharmacological inhibitors, PKCθ mutants, and expression of constitutive active or transdominant negative forms of DGRα, which acts as a negative modulator of DAG levels, to show that tyrosine phosphorylation of and DAG binding to PKCθ are indispensable for selective PKCθ recruitment to the immunological synapse in T lymphocytes.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies used included anti-PKCθ (Transduction Laboratories, Lexington, KY), phycoerythrin-conjugated anti-human CD69, phycoerythrin-conjugated anti-human CD25, purified anti-human CD3 and CD28 (PharMingen, San Diego, CA), anti-phosphotyrosine (clone 4G10; Upstate Biotechnology, Inc., Lake Placid, NY), and horseradish peroxidase-goat anti-mouse IgG (Dako, Glostrup, Denmark). Polystyrene 15-μm microspheres were purchased from Polysciences, Inc. (Warlington, PA); the ECL detection kit (Amersham Biosciences) was used...
according to manufacturer’s instructions. U73122, U73343, calphostin, rottlerin, and PP2 were purchased from Calbiochem; phorbol-12,13-dibutyrate (PDBu), carbachol, bisindolylmaleimide I hydrochloride, and poly-L-lysine were from Sigma.

Recombinant Plasmids—The plasmid encoding EGFP-PKC\(\alpha\) under a pEF promoter was generated by excising PKC\(\alpha\)-EGFP construct (Clontech, Palo Alto, CA) with XhoI and SacII (this end was blunted). The 2.1-kb fragment encoding the PKC\(\alpha\) cDNA was subcloned in the pEGFP-Bos vector (pEGFP-PKC\(\alpha\)) at the EGFP C terminus. To generate mutant constructs, Tyr90 was replaced by Phe or Asp using the QuikChange™ site-directed mutagenesis kit from Stratagene and appropriate oligonucleotides. The kinase-dead and membrane-targeted versions of DGK\(\beta\) fused to EGFP have been described (21).

Cell Culture and Transfection—The JHM1-2.2 cell line was generated by stable transfection of the human muscarinic subtype 1 receptor in the Jurkat leukemic human T cell line (22). Jurkat and JHM1-2.2 cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes, and 100 μg/ml each penicillin and streptomycin (complete medium). Cells in exponential growth were electroporated with 20 μg of DNA constructs using a Gene Pulser (270 V, 975 microfarads; Bio-Rad). Cells were immediately transferred to 30 ml of complete medium and assayed after 24 h.

Immunoprecipitation and Western Blot Analysis—Jurkat T cells (2 \times 10^6) were seeded on 6-well plates precoated with a 1:1 mixture of anti-CD3/CD28 antibody (final concentration 10 μg/ml each) in 50 mM Tris-HCl buffer, pH 8. After 15 min, cells were lysed in Nonidet P-40 buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 200 mM Na_3VO_4, 100 mM NaF, 10 mM NaPP, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) and cleared by centrifugation. Protein lysates (200 μg) were incubated with the indicated antibodies (2 μg, 1 h, 4 °C) followed by protein A-Sepharose (1 h, 4 °C). Immunoprecipitated complexes were washed, and proteins were separated in SDS-PAGE, transferred to nitrocellulose membrane, incubated with the indicated antibody, and developed using the ECL detection kit (Amersham Biosciences) according to the manufacturer’s instructions.

Blood Collection and Lymphocyte Purification—Blood was drawn from healthy volunteers. Buffy coat (40 ml) was overlaid on a Ficoll-
Hypaque gradient (1:2) and centrifuged, and the lymphocyte-containing infranatant band was collected and washed with serum-free RPMI 1640 medium. The cell pellet was suspended in RPMI 1640 with 10% fetal calf serum, and cells were counted in a Neubauer hemocytometer.

**Anti-CD3/CD28 Stimulation and Immunofluorescence**—Cells were harvested 24–36 h after electroporation. For anti-CD3/CD28 stimulation, slides were precoated with a 1:1 mixture of anti-CD3/anti-CD28 antibody (final concentration 10 μg/ml each) in 50 mM Tris-HCl buffer, pH 8. Lymphocytes were plated onto slides and examined by confocal microscopy after 15 min. For pharmacological inhibition, cells were pretreated (30 min) with inhibitors before plating onto anti-CD3/CD28-coated slides. For stimulation with antibody-coated microspheres, antibodies were adsorbed to microspheres by mixing 0.5 μg of antibody in phosphate-buffered saline with 0.5 × 10^6 microspheres (final volume 1 ml) and incubating (1.5 h, room temperature) with continuous mixing; 1.5 ml of 1% bovine serum albumin in PBS was added, and mixing continued (30 min). Microspheres were washed three times with phosphate-buffered saline and resuspended in phosphate-buffered saline. For stimulation, 10^6 transfected cells were mixed with antibody-coated beads at a 2:1 cell/bead ratio and plated on chambered slides. A time series of images was captured by confocal microscopy.

**Analysis of Cell Surface CD69 or CD25 Expression**—Cells were plated in anti-CD3/CD28-coated 6-well plates. Cell surface CD69 or CD25 expression was analyzed 20 h later using phycoerythrin-conjugated anti-human CD69 or phycoerythrin-conjugated anti-human CD25 monoclonal antibody. Immunofluorescence intensity of cells was determined by flow cytometry (EPICS-XL; Beckman Coulter). For inhibitor treatment, cells were incubated with vehicle or inhibitors for 30 min and then plated as above. For analysis of CD69 expression in transfected cells, cells were seeded 24 h after transfection onto anti-CD3/CD28-coated plates, and CD69 expression was analyzed after 6 or 20 h, gating for green fluorescent protein-positive and -negative cells.

**Calcium Determination**—Cells were cultured in complete medium, harvested, centrifuged (5 min, 20,000 × g), and then resuspended in 1 ml of complete medium at 10^6 cells/ml. Fluo-3-AM (10 μM) was added to 10^6 cells (6 μl) and mixed (37 °C, 25 min). Cells were washed twice and resuspended (5 × 10^6 cells/ml) in complete medium containing 2 mM CaCl2 and incubated (37 °C, 5 min), and fluorescence intensity was measured by flow cytometry following anti-CD3/CD28 stimulation.

**RESULTS**

PKCθ is the only PKC isoform recruited to the immunological synapse after TCR stimulation, suggesting that its activation mechanism differs from that of the other isoforms. To assess the changes in subcellular PKCθ location following TCR stimulation in intact T cells, we generated an EGFP-PKCθ construct. When transiently transfected Jurkat cells were stimulated with anti-CD3/CD28-coated polystyrene beads, this construct translocated to the cell-bead contact zone (Fig. 1A), as described for the endogenous PKCθ. To analyze the specificity of the PKCθ recruitment mechanism in response to TCR engagement, protein translocation was evaluated in JHM1-2.2, a Jurkat subcell line stably transfected with the human muscarinic type I receptor. Carbachol stimulation of these cells induces DAG-dependent activation signals through PLC-β activation (22). As in the parental Jurkat line, PKCθ translocated to the membrane when these cells were stimulated with anti-CD3/CD28-coated beads (not shown) or with immobilized anti-CD3/CD28 antibody (Fig. 1B, *upper panel*). Under these conditions, some perinuclear localization was also seen. Cell treatment with the Src-like kinase-specific inhibitor PP2 (23) prevented EGFP-PKCθ translocation after TCR activation. Translocation was also observed after carbachol stimulation, but, in this case, PP2 treatment did not prevent membrane localization of the EGFP-PKCθ construct (Fig. 1B, *lower panel*).

CD69 is a lymphocyte activation marker; its expression is DAG-mediated and correlates with PKCθ activation (17, 24). We thus analyzed PKCθ activation by measuring CD69 expression. Cell surface CD69 expression increased in response to both TCR and human muscarinic type I receptor stimulation (Fig. 1C). As for EGFP-PKCθ membrane translocation, PP2 treatment of cells prevented CD69 expression in response to anti-CD3/CD28 stimulation but did not affect CD69 induction after carbachol stimulation. This validates the use of CD69 expression as an indicator of PKCθ translocation.

To assess the contribution of DAG generation to PKCθ translocation, EGFP-PKCθ translocation was determined in intact Jurkat cells following treatment with various pharmacological inhibitors known to interfere with DAG-based signals. Treatment with the PLC-γ inhibitor U73122 prevented EGFP-PKCθ translocation to the membrane, indicating that PLC-γ-dependent DAG generation is essential for membrane localization (Fig. 2A). The addition of calphostin C, which competes with PKCs for the DAG binding site, also impeded EGFP-PKCθ membrane translocation after TCR engagement (Fig. 2A). Finally, treatment with the PKC inhibitor bisindolymaleimide I hydrochloride (25) did not affect PKCθ translocation (Fig. 2A), indicating that activity of other PKC isoforms is not involved in PKCθ translocation. Although translocation to the plasma membrane is blocked by U73122 and calphostin C, EGFP-PKCθ appears to remain associated to internal membranes (Fig. 2A).

In response to TCR triggering, PKCθ is tyrosine-phosphorylated and can be detected in anti-Tyr(P) immunoprecipitates (26). Inhibition of PLC-γ activation did not alter the amount of PKCθ immunoprecipitated by anti-Tyr(P) after TCR stimulation (Fig. 2B). PLC-γ inhibition thus blocks PKCθ translocation, without affecting Lck-mediated PKCθ Tyr phosphorylation or its recruitment to Tyr-phosphorylated protein complexes. To corre-
late PKCα membrane translocation with its biological activity, we examined CD69 expression after PLC-γ inhibition. Following U73122 treatment, CD69 expression was severely impaired, to an extent similar to that seen after PP2 inhibition (Fig. 2C).

To demonstrate that DAG-dependent PKCα activation is a physiological mechanism in the lymphocyte immune response and is not cell line-specific, similar experiments were performed using peripheral blood lymphocytes (Fig. 3A). Both blockade of PLC-γ activation and treatment with the PKCα-specific inhibitor rottlerin (25) block CD69 up-regulation. This supports the finding that PLC-γ-mediated DAG production also controls CD69 expression in human lymphocytes via PKCα activation. PKCα regulates lymphocyte activation through expression of the transcription factors NF-κB and AP-1 and subsequent induction of the IL-2 and CD25 genes (14, 24, 27, 28). Specific inhibition of PLC-γ or PKCα diminished surface CD25 expression and impaired lymphocyte progression to later activation stages (Fig. 3B).

It has been proposed that PKCα Tyr90 is the phosphorylatable residue in response to TCR activation (26). To assess the specific contribution of Tyr phosphorylation in TCR-elicited PKCα translocation, two EGFP-PKCα mutants were prepared. In one, Tyr90 was replaced by Phe, preventing protein phosphorylation; in the other, Tyr90 was replaced with Asp, which mimics the negative charge of phosphorylated Tyr, simulating phosphorylation at this residue. When analyzed by confocal microscopy, the EGFP-PKCα(Y90F) protein did not translocate to the membrane after TCR stimulation (Fig. 4A).

The EGFP-PKCα(Y90D) mutant was found in cytosol in unstimulated cells, and translocation was observed only after TCR engagement (Fig. 4B). Furthermore, PP2 inhibition of the Lck pathway or U73122 inhibition of DAG generation also blocked EGFP-PKCα(Y90D) translocation following stimulation.

Earlier studies ruled out DAG requirement for PKCα translocation, based on the observation that this protein is found at the membrane after TCR stimulation in a PLC-γ-deficient Jurkat subcell line (19). As these authors showed by fractionation analysis, we observed that PKCα translocated to the plasma membrane when immobilized anti-CD3/CD28 was used as a stimulus (Fig. 5). Western blot analysis of the two PLC-γ isoforms known to be present in T lymphocytes showed that, whereas PLC-γ1 was absent in these cells, PLC-γ2 was present at high levels. Analysis after TCR stimulation demonstrated that, although to a lower extent than in parental Jurkat cells, Ca2+ levels were increased in these PLC-γ1-deficient cells. We confirmed that this Ca2+ increase was PLC-γ-dependent, since it was blocked by U73122 treatment (Fig. 5). TCR-induced Ca2+ release was not blocked by the physiologically inactive analog U73343 (not shown).

In T lymphocytes, clearance of PLC-generated DAG is regulated by DGKα, which converts this lipid to PA (21). To correlate PKCα activation with DAG clearance, we determined CD69 expression in Jurkat cells transiently transfected with a catalytically inactive DGKα form (DGKkd) known to induce higher, sustained DAG levels in response to TCR activation (29). In basal conditions, CD69 expression was up-regulated in transfected compared with untransfected cells (Fig. 6A). After TCR stimulation, CD69 expression was markedly elevated in cells expressing DGKkd, correlating with the increased DAG generation in these cells. Pharmacological inhibition of PLC-γ and Lck returned CD69 expression to basal levels, indicating that the CD69 increase in DGKkd-expressing cells was due to attenuation of endogenous DGK activity and not to further regulation.
We next measured CD69 expression in Jurkat cells transiently transfected with a constitutively active DGK/H9251 construct (Myr-EGFP-DGK/H9251). Myr-EGFP-DGK/H9251 locates at the plasma membrane, attenuating receptor-regulated responses by phosphorylating PLC-γ-generated DAG (21). Accordingly, in Myr-EGFP-DGK/H9251-transfected cells, cell surface CD69 expression was down-modulated compared with untransfected cells (Fig. 6B).

To confirm the role of DGK/H9251 as a negative modulator of PKC/H9258 relocalization, we studied TCR-induced endogenous PKC/H9258 translocation in Myr-EGFP-DGK-transfected Jurkat cells. Endogenous PKC/H9258 translocation to the membrane was detected by confocal microscopy following TCR triggering or after the addition of the DAG analog PDBu to the cells (Fig. 6C). In TCR-stimulated Myr-EGFP-DGK-expressing cells, PKC/H9258 translocation from cytosol to the membrane was impaired (Fig. 6D). Myr-EGFP-DGK expression did not affect PDBu-induced membrane translocation of PKC/H9258, showing that only TCR-induced signals were affected.

**DISCUSSION**

PKC/H9258 translocation to the T cell synapse, which is formed when a major histocompatibility complex-bound peptide in APCs contacts specific T cells, is crucial for subsequent T cell activation (13). Following TCR/CD28 costimulation, PKC/H9258 activates IKKγ, leading to NF-κB activation; this effect is T cell-specific, since PKC/H9258 does not activate NF-κB efficiently in nonlymphoid cells (28). In mature primary T cells, the NF-κB cascade is the major and physiologically most important target of PKC/H9258 in the TCR/CD28 costimulatory pathway leading to IL-2 production.

Receptor-induced PKC translocation to membranes is a complex process that involves protein phosphorylation, protein-lipid, and protein-protein interactions (30, 31). Here we used several biochemical and genetic approaches to show that TCR-mediated PKC/H9258 translocation to the cell membrane requires an integrated two-step signal involving PKC/H9258 Tyr phosphorylation as well as DAG generation at the plasma membrane.

The use of JHM1–2.2, a Jurkat-derived T lymphoid cell line stably transfected with the human muscarinic type I receptor, demonstrates that, through activation of distinct PLC isoforms, both TCR and G protein-coupled receptors can induce PKC/H9258 translocation. Nonetheless, experiments using the Lck inhibitor PP2 indicate that Src tyrosine kinase-imposed regulation is restricted to TCR-induced PKC/H9258 translocation, which implies receptor-dependent selective pathways.

Activation of the Src tyrosine kinases p56Lck and p59Fyn is a very early event in the TCR-triggered signaling cascade (2). Cell treatment with PP2 thus affects the phosphorylation/activation of several molecules including different types of adapters and signaling mediators, among them PLC-γ-1. When DAG
FIG. 6. DGKε-dependent regulation of PKCθ translocation and activation in response to TCR. A, expression of a catalytically inactive DGKε in Jurkat cells correlates with increased CD69 cell surface expression. Jurkat cells were transfected with the GFP-DGKε K<sub>2</sub> construct. After 24 h, cells were seeded onto anti-CD3/CD28-coated dishes. CD69 cell surface expression was analyzed 20 h later by FACSscan analysis of GFP<sup>+</sup> and GFP<sup>−</sup> gated cells. The results are displayed as histograms of CD69-positive cells in the two populations. Where indicated, cells were pretreated with inhibitors for 30 min prior to stimulation. B, expression of constitutive active DGKε in Jurkat cells correlates with decreased cell surface CD69 expression. Jurkat cells were transfected with Myr-EGFP-DGKε and, after 24 h, were stimulated with anti-CD3/CD28 antibodies. After 20 h, CD69 cell surface expression was analyzed in GFP<sup>−</sup> and GFP<sup>+</sup> gated cells. The results are displayed as histograms of CD69-positive cells in both populations. C, translocation of endogenous PKCθ. Jurkat cells were seeded onto anti-CD3/CD28-coated slides, and translocation of endogenous PKCθ was analyzed by confocal microscopy after 10 min. As a translocation control, cells were stimulated with PDBu (200 nM, 15 min). D, constitutive membrane localization of DGKε prevents TCR-induced membrane translocation of endogenous PKCθ. Jurkat cells were transfected with Myr-EGFP-DGKε and, after 48 h, were stimulated with anti-CD3/CD28 antibodies or PDBu (200 nM, 15 min), and membrane localization of endogenous PKCθ was determined by confocal microscopy.

Previous studies suggested that phosphorylation on Tyr<sup>90</sup> in PKCθ was a direct consequence of TCR stimulation (28). Here we confirm the importance of phosphorylation at this residue, showing that substitution of Tyr<sup>90</sup> by Phe prevents anti-CD3/CD28-induced PKCθ translocation to the plasma membrane. Tyr<sup>90</sup> is not located within a consensus binding sequence for Src homology 2 domains (33), suggesting that Tyr<sup>90</sup> phosphorylation may induce a conformational change. This could allow PKCθ binding to DAG by exposing the C1 domains. Tyr<sup>90</sup> mutation would thus prevent PKCθ translocation by rendering PKCθ(Y90F) unable to interact with DAG.

Studies with the Asp<sup>90</sup> mutant, which mimics Tyr<sup>90</sup> phosphorylation, indicate that phosphorylation of this residue, albeit necessary, is not sufficient for TCR-induced PKCθ translocation and that an accessory signal is required. The disruption of anti-CD3/CD28-induced PKCθ translocation following pharmacological inhibition of PLC-γ suggests that PLC-γ-mediated DAG generation constitutes this additional membrane targeting signal.

Pharmacological inhibition of PLC-γ blocked PKCθ membrane translocation but did not alter its Tyr phosphorylation or its recruitment to Tyr-phosphorylated protein complexes. Under these conditions, PKCθ appears to be associated to internal membranes rather than being returned to cytosol. Other DAG-regulated proteins were shown to translocate to internal localizations through protein-protein interactions; thus, β-COP, a Golgi protein, acts as a cellular receptor for PKCε (34), whereas the βγ chimerin-interacting protein Tmp21 is found in the Golgi and endoplasmic reticulum (35). By inducing a conformational change in the absence of membrane DAG generation, Tyr phosphorylation might thus allow PKCθ association with cytoskeletal and/or internal membrane-associated proteins. PLC-γ-dependent DAG generation would thus be necessary to ensure correct PKCθ association with the membrane after Tyr phosphorylation. It is important to note that if the absence of DAG caused PKCθ to remain bound to a cytoskeletal or particulate fraction, a fractionation assay would not distinguish this localization from association with the plasma membrane. This may clarify the previously reported PKCθ association to the particulate fraction in the absence of PLC-γ1 activation (19).

The DAG generation requirement for TCR-induced PKCθ membrane recruitment is further confirmed by the use of both constitutive active and transdominant negative DGKα forms. This lipid kinase phosphorylates DAG to PA, acting as a negative modulator of DAG-regulated signals (21). The mutants used here are known to modify existing DAG levels without affecting DAG generation and showed the negative role of DGKα in RasGRP-induced Ras activation (29). As previously demonstrated for RasGRP, we show that plasma membrane PKCθ localization is also negatively regulated by DGKα, confirming the role of this lipid kinase as a modulator of the intensity and duration of TCR-derived signals.

It was recently reported that selective PKCθ recruitment to the TCR engagement site may operate via a phosphatidylinositol 3-kinase-dependent, PLC-γ1/DAG-independent mechanism (19). These authors used PLC-γ1-deficient Jurkat cells to show
PKCθ translocation to the membrane. A model was suggested in which phosphatidylinositol 3-kinase acting upstream of the Vav/Rac pathway would regulate PKCθ translocation via cytoskeletal rearrangement (15, 17, 19). The Vav/Rac pathway reorganizes the T cell actin cytoskeleton and facilitates TCR cytoskeletal rearrangement (15, 17, 19). The Vav/Rac pathway engagement site would in turn ensure PKCθ-mediated DAG generation at the TCR stimulation site. PLCγ translocation is essential for PKC activation, demonstrating that PLCγ isoforms may have an overlapping role in lymphocytes and that these PLCγ-deficient cells cannot be used to rule out a DAG requirement in PKCθ translocation. The phenotype analysis of Vav−/− mice showed disruption of PLCγ activation, demonstrating that PLCγ is one of the main effectors of Vav activation (20). Together, these data suggest that Vav-induced, PKCθ-mediated lymphocyte activation requires PLCγ for PKCθ translocation. Experiments are currently under way to analyze the functional relationship between the Lck/PLCγ and phosphatidylinositol 3-kinase/Vav pathways in spatio-temporal regulation of PKCθ translocation.

DAG generation during T cell activation is a key step in Ras/extracellular signal-regulated kinase pathway initiation through RasGRP translocation (29). Here we report the essential role of DAG in PKCθ membrane localization. Altogether, these conclusions allow us to suggest that PLCγ-mediated DAG generation at the T cell synapse has an essential role in the polarized recruitment of signaling molecules during T cell activation. Whereas Tyr phosphorylation of adaptors and cytoskeletal reorganization ensure relocation of signaling proteins to the T cell synapse, the local increase in DAG levels at the membrane is an essential step to allow rapid recruitment of C1-containing proteins to the proximity of the TCR. A model can be envisaged (Fig. 7) in which Lck-triggered phosphorylation of several effectors, together with Vav/Rac-induced dynamic cytoskeletal reorganization, activates PLCγ to increase local DAG levels. Simultaneously, Tyr phosphorylation of PKCθ promotes a conformational change, exposing its C1-DAG-binding domains that anchor the protein to the membrane. Fine control of PKC activation requires that the elevation in DAG levels be rapid and transient. This is regulated by DGKinase translocation to the membrane, where the lipid kinase decreases membrane DAG levels by phosphorylating DAG to PA, dissociating PKCθ, and terminating the signal. DAG generation at the site of the immunological synapse allows the spatio-temporal protein relocalization that ensures signal transmission during a T cell response.

Acknowledgments—We thank Dr. A. Weiss and Genentech Inc. for providing the JHH1-2.2 cell line, Dr. Abrahams for PLCγ1 deficient cells, the members of the group of I. M. for stimulating discussion, M. C. Moreno-Ortiz for flow cytometry, and C. Mark for excellent editorial assistance.

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