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Lck-Dependent Tyrosine Phosphorylation of Diacylglycerol Kinase α Regulates Its Membrane Association in T Cells[1]

Ernesto Merino,[2]* Antonia Ávila-Flores,[2]* Yasuhiro Shirai,[†] Ignacio Moraga,* Naokai Saito,[†] and Isabel Mérida[3]*

TCR engagement triggers phospholipase Cγ1 activation through the Lck-ZAP70-linker of activated T cell adaptor protein pathway. This leads to generation of diacylglycerol (DAG) and mobilization of intracellular Ca^{2+}, both essential for TCR-dependent transcriptional responses. TCR ligation also elicits transient recruitment of DAG kinase α (DGKα) to the lymphocyte plasma membrane to phosphorylate DAG, facilitating termination of DAG-regulated signals. The precise mechanisms governing dynamic recruitment of DGKα to the membrane have not been fully elucidated, although Ca^{2+} influx and tyrosine kinase activation were proposed to be required. We show that DGKα is tyrosine phosphorylated, and identify tyrosine 335 (Y335), at the hinge between the atypical C1 domains and the catalytic region, as essential for membrane localization. Generation of an Ab that recognizes phosphorylated Y335 demonstrates Lck-dependent phosphorylation of endogenous DGKα during TCR activation and shows that pY335DGKα is a minor pool located exclusively at the plasma membrane. Our results identify Y335 as a residue critical for DGKα function and suggest a mechanism by which Lck-dependent phosphorylation and Ca^{2+} elevation regulate DGKα membrane localization. The concerted action of these two signals results in transient, receptor-regulated DGKα relocalization to the site at which it exerts its function as a negative modulator of DAG-dependent signals. ***The Journal of Immunology,*** **2008, 179: 0000–0000.**

Activation of T lymphocytes in response to foreign Ags is essential for an adequate immune response to tumors and infection (1). The mechanisms governing T cell activation require fine-tuning, because T cell activation defects induce immunosuppression and anergy, whereas hyperactivation can lead to immunoproliferative disorders and autoimmunity. Peripheral T cells are activated following TCR triggering in the presence of costimulatory signals (2). This initiates the concerted activation of several protein tyrosine kinases (PTK)[4] that, in turn, promote assembly of signaling complexes, leading to second messenger generation and cytoskeletal reorganization (3, 4).

The Src kinase family members $p56^{lck}$ (Lck) and $p59^{fyn}$ (Fyn) are the major PTK activated during TCR triggering. These kinases phosphorylate the ITAM motifs in CD3 and TCRζ, enabling binding of ZAP70 (ζ-chain TCR-associated protein kinase 70 kDa), which subsequently phosphorylates linker of activated T cell adaptor protein (LAT). Phosphorylated LAT provides a docking site for an array of proteins involved in TCR signaling (5, 6). Although Fyn and Lck are closely related, each is predicted to exert discrete, unique functions (7). In fact, Lck (but not Fyn) is primarily responsible for initiating phospholipase Cγ1 (PLCγ) phosphorylation in peripheral T cells (8).

The activation of PLCγ must be accurate, because this enzyme is responsible for generating inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), two essential mediators in the initiation and maintenance of the T cell activation program (9, 10). IP_3 mediates an increase in intracellular Ca^{2+} ($[Ca^{2+}]i$) levels and ensures accurate activation of NF-AT-modulated genes (11, 12), whereas DAG generation at the membrane regulates localization and activation of several signaling molecules, including protein kinase C (PKC)θ, Ras-GRP1, and protein kinase D (3, 13). DAG- and $[Ca^{2+}]i$-regulated signals are both necessary for an appropriate immune response; in addition, an adequate balance between these two messengers guarantees correct initiation and termination of the T cell activation program. Accordingly, Ca^{2+} flux generation in the absence of sufficient DAG is proposed to lead to anergy (14, 15), whereas defects in DAG signal termination are linked to lymphoproliferative disease and/or autoimmunity (16, 17). The requirement for mechanisms to oversee DAG consumption thus emerges as an important aspect in T cell response control.

The DAG kinases (DGK) are a family of signaling proteins that modulate DAG levels by catalyzing its conversion into phosphatidic acid (PA) (18–21). Mammalian cells express five DGK subtypes, characterized by the presence of a common catalytic domain

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[4] Abbreviations used in this paper: PTK, protein tyrosine kinase; $[Ca^{2+}]i$, intracellular Ca^{2+}; DAG, diacylglycerol; DGK, DAG kinase; HA, hemagglutinin; IP_3, inositol 1,4,5-triphosphate; KD, kinase dead; LAT, linker of activated T cell adaptor protein; MFI, mean fluorescence intensity; PA, phosphatidic acid; PKC, protein kinase C; PLCγ, phospholipase Cγ1; pY, proteins phosphorylated in tyrosine residues; SH, Src homology; SHP-1, SH region 2 domain-containing phosphatase-1; wt, wild type.

and at least two protein kinase C-like type 1 (C1) domains. In addition, each DGK subtype has distinct regulatory motifs, suggesting the existence of diverse regulatory mechanisms and/or participation in different signaling complexes. DGKα is a type I DGK characterized by two EF hand Ca^{2+}-binding domains (22) and a recoverin-like domain in the N-terminal region (23) that is abundantly expressed in the thymus and mature T cells (24, 25). Early studies in T cells demonstrated that, during T cell activation, DGKα located at the cytosol in resting T cells translocates to the membrane (24). DGKα membrane localization and activation act as a switch-off signal for Ras activation, mediated by localization to the membrane of Ras-GRP1 (24, 26). The recent generation of DGKα-deficient mice confirmed these results, showing that stimulation of DGKα-null T cells elicits increased RasGTP levels and MAPK activation (17). Together, these studies indicate that DGKα controls the magnitude of the TCR response, acting as a brake at the initial steps of TCR signaling.

Like most DGK, DGKα must translocate to the membrane to exert its regulatory function. Studies using GFP-DGKα fusion proteins and the Jurkat T cell model have allowed a detailed analysis of the signals required for receptor-dependent translocation (27). The DGKα N-terminal domain acts as a negative regulator of enzyme localization, maintaining the enzyme in a cytosolic/inactive conformation unless modified by receptor-derived $[Ca^{2+}]i$ to an active/membrane-bound conformation. Nonetheless, in T lymphocytes, Ca^{2+} mobilization is necessary, but not sufficient, to induce DGKα localization to the plasma membrane unless PTK are also activated. This suggested a more complex mechanism by which PTK-dependent signaling is required to regulate DGKα membrane localization (27). Additional studies in lymphoid and nonlymphoid cells point to PTK-dependent regulation of this isoform. IL-2 was shown to activate DGKα by a Ca^{2+}-independent mechanism that requires PTK-mediated PI3K activation (28, 29). Studies in nonlymphoid cells suggest that Src kinase-dependent phosphorylation is necessary to promote DGKα activation (30). There are nonetheless no reports of direct tyrosine phosphorylation of endogenous DGKα in response to physiological stimulation.

In this study, we investigated DGKα regulation by tyrosine phosphorylation during T cell activation. We found that TCR triggering induces tyrosine phosphorylation of endogenous DGKα by a mechanism dependent on Lck. Because previous studies identified Y335 as a residue phosphorylated in a Src-dependent manner in nonlymphoid cells, we examined phosphorylation of this residue using a phospho-Y335-specific Ab. We found that endogenous DGKα is phosphorylated at Y335 and that this phosphorylation is not observed in cells lacking Lck. TCR triggering induces rapid, transient elevation of Y355 phosphorylation, and fractionation analysis showed that phosphorylated DGKα localized specifically at the membrane. These results suggest that phosphorylation at Y335 stabilizes DGKα membrane localization. Accordingly, the use of a nonphosphorylatable mutant showed that this tyrosine is essential for DGKα translocation to the membrane, where it exerts its function. This is the first description of tyrosine phosphorylation of endogenous DGKα in T lymphocytes as a mechanism to modulate membrane localization of the enzyme and, thus, to attenuate DAG-dependent signals.

Materials and Methods

Cell culture

The human leukemia Jurkat T cell line, the Lck-defective Jurkat variant JCaM1 (31), and the kidney epithelial cell line HEK293 were maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen) and 2 mM L-glutamine (37°C, 5% CO_2). BaF/3 cells and the human leukemia Molt4 cell line were maintained in RPMI 1640 (Invitrogen) supplemented as above; BaF/3 cell medium also contained 5×10^{-5} M 2-ME and 5% WEHI-3B supernatant as an IL-3 source. Human PBL were prepared from buffy coats using a Ficoll density gradient. T lymphocyte purity was >90%, as analyzed by flow cytometry using the T3b anti-CD3 mAb.

Abs and reagents

Polyclonal rabbit anti-Lck, anti-PLCγ, and anti-phosphotyrosine (clone AG10) mAbs were from Upstate Biologicals; anti-Lck, anti-CD3, and anti-CD28 mAb were from BD Pharmingen. Mouse anti-MAPK (ERK1 plus ERK2) was from Zymed Laboratories; anti-pMAPK (ERK1 plus ERK2), anti-pIκBα, anti-IκBα, anti-pPLCγ (Tyr^{783}), and anti-p(serine) PKC substrate were from Cell Signaling Technology; anti-GFP mAb was from BD Clontech; anti-actin (clone AC-15), anti-vimentin, and anti-α-tubulin were from Sigma-Aldrich; HRP-coupled polyclonal goat anti-mouse and anti-rabbit Ig were from DakoCytomation; rabbit anti-mouse IgG was from Jackson ImmunoResearch Laboratories; and anti-hemagglutinin (HA) Ab was from Covance. The DGKα-specific mouse mAb mixture was a gift from W. van Blitterswijk (Netherlands Cancer Institute, Amsterdam, The Netherlands). Mouse mAb against the Myc tag and rabbit anti-NF-AT Ab were provided by P. Hawkins (Babraham Institute, Cambridge, U.K.) and J. Redondo (CNIC, Madrid, Spain). PMA, leupeptin, and aprotinin were from Sigma-Aldrich. Ionomycin, DGK inhibitor R59949, PI3K inhibitor LY294002, and the Src PTK family inhibitor PP2 were from Calbiochem. γ-binding Sepharose was from GE Healthcare.

Plasmids and transfections

Plasmid encoding human DGKα fused to Myc (Myc-DGKα) was a gift from A. Graziani and has been previously described (30); plasmids encoding Lck and a constitutive active version bearing Y505 to F mutation (Lck^{505}) were a gift from A. Carrera (29); plasmids encoding DGKα or a catalytically inactive mutant fused to GFP (GFP-DGKα, GFP-DGKαKD) or to HA (HA-DGKα) were previously described (27, 29). DGKα and DGKαKD mutants Y335F were generated using the Quickchange site-directed mutagenesis kit (Stratagene) and the following primers: 5′-CCTC CATCTTCCATCTTTCCCAGTGTCCTGGCC-3′ and 3′-GGAGGTAGA AGGTAGAAAGGGTCACAGGACCGG-5′.

For transfection, lymphocytes in logarithmic growth were electroporated with 25 μg of plasmid DNA. HEK293 cells were transfected with LipofectAmine (Invitrogen). All experiments were performed 24 h after transfection.

Cell stimulation, lysis, and Western blot

Jurkat T cells were washed once with DMEM, then starved (1 h) or resuspended immediately in complete medium (5×10^6 cells/ml). Cells were stimulated with anti-CD3 alone or with anti-CD28 mAb (1 μg/ml each). For TCR cross-linking, 10^7 cells were resuspended in 250 μl of complete medium and incubated (10 min, on ice), after which 2.5 μg each of anti-CD3 and anti-CD28 was added and the mixture incubated (45 min, on ice). Cells were washed twice with cold medium and resuspended in 250 μl of cold medium containing 7.5 μg of rabbit anti-mouse IgG Ab. Cells were incubated for the indicated times at 37°C, pelleted, and submitted to subcellular fractionation. Where indicated, cells were incubated (37°C, 5% CO_2, 1 h) with PI3K inhibitor LY294002 (10–40 μM), DGK inhibitor R59949 (30 μM), or the Src PTK family inhibitor PP2 (1–20 μM). In some cases, cells were treated with PMA (200 nM) and/or ionomycin (1 μM) plus 2 mM $CaCl_2$ as a Ca^{2+} source. PBL were stimulated with anti-CD3 mAb or anti-CD3/CD28 mAb (1 μg/ml each) for the times indicated.

After treatment, cells were lysed immediately in ice-cold lysis buffer (10 mM HEPES (pH 7.5), 15 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.2% Nonidet P-40, 1 mM DTT, 50 mM NaF, 10 μg/ml each leupeptin and aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, and 20 mM glycerol phosphate) by incubation with gentle rocking (20 min, 4°C). Cell lysates were centrifuged ($15{,}000 \times g$, 15 min, 4°C), and supernatant proteins were resolved by SDS-PAGE. Gels were blotted onto nitrocellulose filters, which were incubated with the indicated Abs diluted in TBST containing either 5% milk or 5% BSA (4°C, overnight). After incubation with secondary Ab (room temperature, 1 h), blots were visualized using ECL (Amersham Biosciences). Where indicated, pY335DGKα bands were quantified by analysis of films using the Image J Program, and values were normalized against the corresponding total DGKα protein band.

Immunoprecipitation

Cells were collected and lysed in ice-cold Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM PMSF, 10 ng/μl aprotinin, and 10

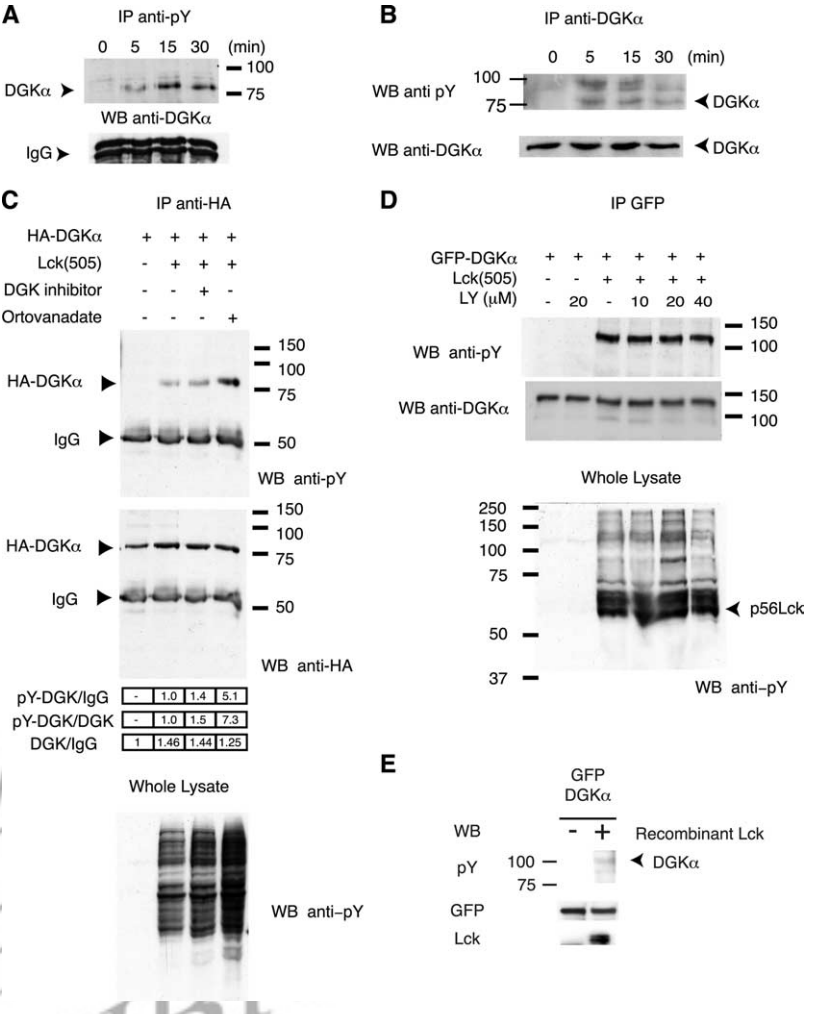

FIGURE 1. DGKα is tyrosine phosphorylated in response to TCR stimulation. *A*, Western blot analysis of DGKα in anti-PY immunopellets obtained from Jurkat cells stimulated at times indicated with anti-CD3. *B*, Same as in *A*, except than DGKα was immunoprecipitated using anti-DGKα and the blot was probed with anti-pY and then with anti-DGKα. *C*, Lck-dependent phosphorylation of DGKα. BaF/3 cells were cotransfected with a plasmid encoding HA-DGKα and an empty plasmid or a plasmid encoding an active $p56^{Lck}$ mutant (p56Lck 505). At 24 h, cells were untreated or incubated (1 h) with R59949 (5 μM) or orthovanadate (1 mM). HA-DGKα was immunoprecipitated using anti-HA. Immunoprecipitated proteins and 20 μg of total cell lysates were resolved by SDS-PAGE, blotted, and probed with the indicated Abs. Tyrosine phosphorylation in total cell lysates (*bottom panel*) was determined as a control of $p56^{Lck}$ expression/activity. *D*, BaF/3 cells were cotransfected as above, then left untreated or incubated with the PI3K inhibitor LY294002 (2 h) at the concentrations indicated. GFP-DGKα was immunoprecipitated with anti-GFP. Blots were probed with anti-pY mAb and reprobed with anti-DGKα mAb to determine total expression levels. Tyrosine phosphorylation was determined in total cell lysates (*bottom panel*) as an indicator of $p56^{Lck}$ expression/activity. *E*, GFP-DGKα was immunoprecipitated from transfected HEK293 cells and subjected to an in vitro phosphorylation reaction without further addition or in the presence of rLck. Proteins were resolved in SDS-PAGE and probed with anti-pY Ab. All experiments are representative of three or four different experiments performed with similar results.

ng/μl leupeptin). Cell lysates were centrifuged (15,000 $\times$ *g*, 15 min, 4°C) and subsequently quantified.

DGKα and the pY proteins were immunoprecipitated by incubating cell lysates with the appropriate Ab (2 h, 4°C). Complexes were then precipitated by addition of γ-binding Sepharose beads (1 h, 4°C). Bead-bound complexes were washed three times in lysis buffer, once with 0.5 M LiCl, and twice with 150 mM Tris (pH 7.5). Finally, beads were resuspended in Laemmli buffer and analyzed by SDS-PAGE and immunoblot.

For in vitro kinase assay, purified GFP-DGKα was incubated with or without 0.25 μg of rLck (MBL) in 50 μl of reaction buffer (50 mM HEPES (pH 7.5), 10 mM $MnCl_2$, 0.01% Triton X-100, and 2.5 mM DTT) for 5 min at 30°C. In vitro phosphorylation reaction was inititated by ATP (500 μM) addition; after 5 min at 30°C, the reaction was stopped by adding loading buffer. Samples were analyzed by SDS-PAGE.

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DGK assay

DGK activity was determined by measuring radioactive phosphate incorporation into PA, using C8-DAG as substrate, as described (28). The reaction was conducted for 10 min at room temperature, followed by lipid extraction using $CHCl_3$/MeOH/2 N HCl (20:10:5, v/v/v). Dried radioactive-labeled lipid products were dissolved in 40 μl of $CHCl_3$/MeOH (1:1, v/v) and applied to a silica gel thin-layer chromatography plate, with unlabeled C8-PA as a migration standard. Plates were developed in a chloroform/methanol/4 M ammonia solvent system (9:7:2, v/v/v) and autoradiographed.

Subcellular fractionation

Fractionation was performed, as described (32), with some modifications. Briefly, cells were harvested and resuspended in cold lysis buffer 1 (5 mM Tris-HCl (pH 7.5), 10 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 40 μg/ml digitonin) supplemented with a mixture of protein inhibitors and lysed (15 min, on ice). After centrifugation (4500 $\times$ *g*, 4 min, 4°C), supernatants (cytosolic fraction, C) were collected and pellets were resuspended in cold lysis buffer 2 (as for buffer 1, with 0.2% Nonidet P-40 instead of digitonin) and lysed (10 min, on ice). After centrifugation (15,000 $\times$ *g*, 15 min, 4°C), supernatants (membrane fraction 1, M1) were collected. The pellet was further extracted with lysis buffer 3 (as for lysis buffer 2, with 1% Nonidet P-40). The supernatant contained membrane-associated proteins and was designated as M2. The pellet was solubilized in Laemmli buffer and corresponds to the cytoskeleton proteins. The different fractions were resolved in SDS-PAGE and analyzed by immunoblot.

Immunofluorescence microscopy

Jurkat cells were harvested 24 h after transfection, washed once, and resuspended in HEPES balanced solution. The cell suspension was transferred to chambered coverslips coated with anti-CD3/CD28 mAb (final concentration 5 μg/ml, 4°C, overnight). Cells were imaged with a laser-scanning confocal microscope (TSC-NT; Leica Microsystems).

Analysis of cell surface CD69 expression

CD69 expression on the cell surface of the GFP-positive population was analyzed 24 h after transfection using a PE-conjugated anti-human CD69 mAb. Cells were stimulated with anti-CD3/CD28 mAb (0.2 μg/ml) for 2.5 h, and immunofluorescence intensity of the cells was determined by flow cytometry (Excalibur; BD Biosciences). Flow cytometry data are presented both as histograms and as plots of mean fluorescence intensity (MFI) of CD69, normalized to that of the GFP-negative population within each sample, against the mean fluorescence intensity of GFP.

Generation of the pY335 Ab

A synthetic peptide corresponding to the porcine DGKα sequence (NH2-CPPSSI(phospho-Y)PSVLA-COOH) was conjugated to keyhole limpet hemocyanin, and 300 μg of keyhole limpet hemocyanin-conjugated Ag emulsified with CFA was injected into a 10-wk-old female rabbit (Japanese

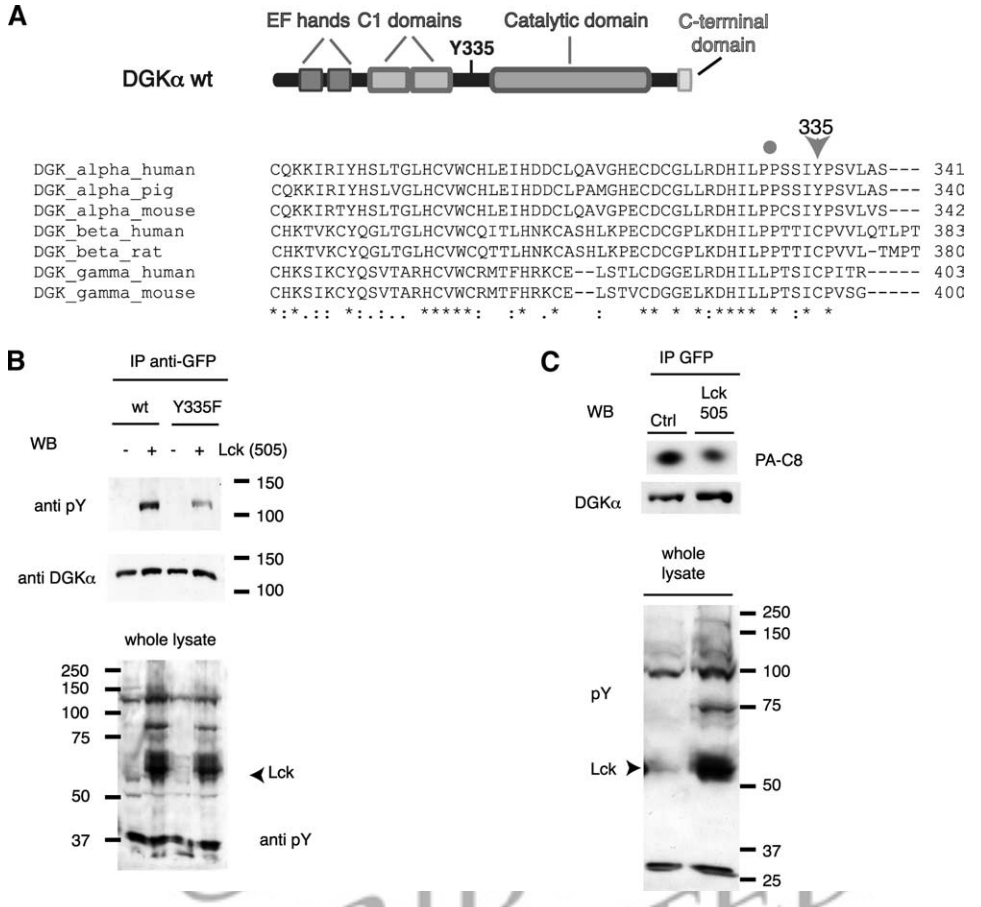

FIGURE 2. *A*, Sequence comparison of the type I DGK family. *Top*, The cartoon represents the modular organization of DGKα and the localization of Y335. *Bottom*, An alignment of different DGKα orthologues indicates the conservation of Y335 (arrowhead). A proline region probably important as a hinge for DGKα folding is also depicted (dot line). *B*, Lck phosphorylates DGKα at Y335. BaF/3 cells were cotransfected with a plasmid encoding GFP-DGKα wt or GFP-DGKα Y335F mutant, together with an empty plasmid or a plasmid encoding Lck, as indicated. GFP-DGKα was immunoprecipitated using anti-GFP, proteins were resolved by SDS-PAGE, and blots were probed with the indicated Ab. Tyrosine phosphorylation was determined in total cell lysates as a control of Lck expression/activation. *C*, Lack of Y335 phosphorylation impairs Lck-dependent DGKα activation. BaF/3 cells were transfected with plasmids encoding GFP-DGKα Y335F and Lck where indicated. DGKα activity was determined in the immunopellets by P^{32} incorporation into C8-DAG, as described in *Materials and Methods*. An autoradiogram of the TLC shows the radioactivity comigrating with the C8-PA standard. One-third of the immunopellet was analyzed by SDS-PAGE and immunoblot with anti-DGKα mAb to determine DGKα expression. Tyrosine phosphorylation was determined in total cell lysates as a control of Lck expression/activation.

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White). Booster injections (150 μg of Ag with IFA) were given at 2-wk intervals. Three days after the sixth boost, the rabbit was bled and antiserum was collected. Ab specificity for phosphotyrosine was confirmed by dot and Western blotting, using nonphosphorylated peptide (NH2-CPPSSIYPSVLA-COOH) and the Y334F mutant of porcine DGKα as negative controls.

Results

Endogenous DGKα is tyrosine phosphorylated in response to TCR stimulation

TCR triggering in T lymphocytes is rapidly followed by activation of cytosolic PTK (33), which in turn phosphorylate several signaling proteins and adaptors (10). Our previous results demonstrated that DGKα translocation to the plasma membrane requires $[Ca^{2+}]$i and the action of PTK (27). To determine whether endogenous DGKα is present in tyrosine-phosphorylated complexes, we mimicked TCR triggering by stimulating Jurkat T cells with anti-CD3 mAb (34) for different time periods; proteins phosphorylated in tyrosine residues (pY) were then purified from cell lysates by immunoprecipitation with anti-pY mAb. Endogenous DGKα was readily detected in the anti-pY complex from 5 min poststimulation until the latest time tested (30 min; Fig. 1*A*).

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Detection of DGKα in anti-pY pellets suggested tyrosine phosphorylation of the protein, although it could also reflect DGKα association to tyrosine-phosphorylated proteins. To analyze the effect of TCR triggering on DGKα tyrosine phosphorylation, we purified endogenous protein by immunoprecipitation and assessed phosphorylation by immunoblot using anti-pY mAb. We observed transient phosphorylation of DGKα on tyrosine residues, with a maximum from 5 to 15 min poststimulation, followed by a decrease (Fig. 1*B*).

DGKα is tyrosine phosphorylated by Lck

Previous experiments in endothelial cells suggested Src-dependent phosphorylation of DGKα (30). In T lymphocytes, the Src kinase family members Lck and Fyn initiate signaling events downstream of TCR stimulation (31). In contrast to Lck, Fyn expression was reported to be very low in Jurkat cell lines (35); we therefore focused on the action of Lck. To study the role of this PTK on DGKα phosphorylation, we analyzed phosphorylation of ectopically expressed DGKα in BaF/3 cells. This proB cell line lacks endogenous Lck, but expresses other Src family PTK, such as Fyn

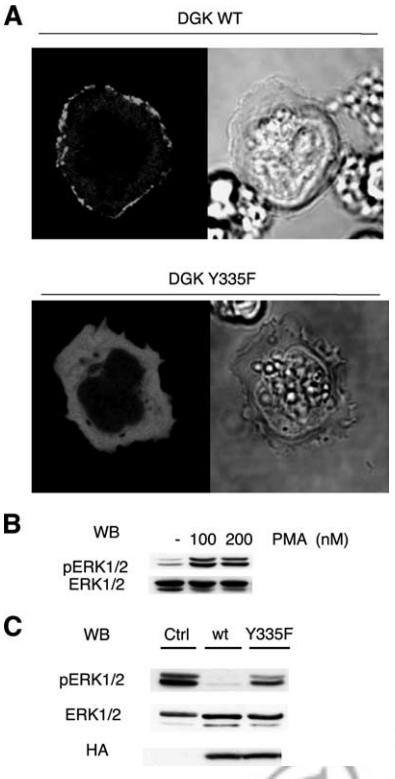

FIGURE 3. DGKα Y335 phosphorylation is essential for DGKα function. *A*, DGKα Y335F mutant does not translocate to plasma membrane following TCR stimulation. Jurkat cells were transfected with either GFP-DGKα or GFP-DGKα Y335F mutant. Twenty-four hours after transfection, cells were plated onto anti-CD3/anti-CD28-coated plates, and the subcellular localization of the GFP-fused proteins was determined by confocal microscopy. *B*, PMA-dependent ERK phosphorylation in HEK293 cells. HEK293 cells were left untreated or stimulated with PMA (1 h). Cells were lysed, and ERK1/2 phosphorylation was determined by analysis of total cell lysates with anti-pERK1/2-specific Ab. *C*, The DGKα Y335F mutant does not attenuate ERK phosphorylation. HEK293 cells were transfected with a plasmid encoding HA-DGKα, the HA-DGKα Y335F mutant, or empty vector. ERK1/2 phosphorylation was determined as in *B*. The same membrane was blotted with anti-HA Ab as a control of protein transfection.

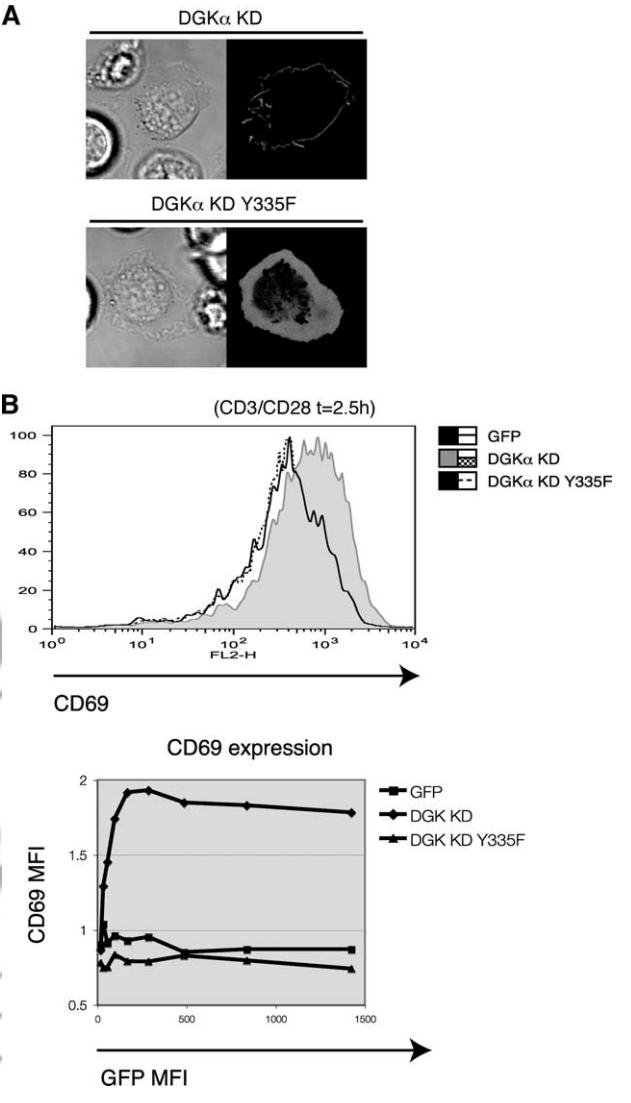

FIGURE 4. Y335 phosphorylation is required for the dominant-negative properties of DGKα KD. Jurkat cells were transfected with either DGKα KD or DGKα KD Y335F mutant fused to GFP. *A*, The subcellular localization of the GFP-fused proteins was determined as in Fig. 3. *B*, CD69 expression was determined by flow cytometry after 2.5-h stimulation with CD3/CD28. At the *top*, analysis of CD69 expression in the GFP-positive population is shown. At the *bottom*, CD69 MFI was plotted vs GFP MFI. The results shown are from a single experiment representative of three performed with similar results.

and Lyn (36). In this cell line, we previously showed that constitutive active Lck induces elevated DGKα activity (29). We observed tyrosine phosphorylation of ectopically expressed DGKα only when BaF/3 cells were cotransfected with a plasmid encoding a constitutive active Lck form (Fig. 1*C*). Cell pretreatment with the pharmacological DGKα inhibitor R59949 did not alter enzyme phosphorylation on tyrosine, suggesting that Lck-dependent phosphorylation of DGKα was independent of enzyme activity. Orthovanadate pretreatment of the cells further increased the level of protein phosphorylation on tyrosine. Our earlier studies suggested that whereas TCR triggering promotes DGKα localization to the plasma membrane (27), IL-2 induces perinuclear localization of this protein and its activation by a PI3K-dependent mechanism (28, 29). Accordingly, inhibition of PI3K activity did not alter Lck-dependent phosphorylation of DGKα (Fig. 1*D*), suggesting that these two pathways represent independent DGKα regulatory mechanisms. Finally, an in vitro kinase assay using purified Lck confirmed direct phosphorylation of DGKα (Fig. 1*E*).

Identification of Y335 as a DGKα phosphorylation site

Amino acid sequence alignment of human, porcine, murine, and rat DGKα orthologues shows a high degree of conservation of Y335 (numbering based on the human sequence), located at the hinge between the second C1 domain and the catalytic domain (Fig. 2*A*). This residue, which is not present in the other two type I isoforms (DGKβ and DGKγ), was recently proposed to be phosphorylated by Src in nonhematopoietic cells in response to hepatocyte growth factor (37) or α-D-tocopherol (38). We therefore studied the role of this residue in Lck-dependent DGKα phosphorylation in hematopoietic cells. BaF/3 cells were transiently cotransfected with a plasmid that coded for Lck and a plasmid encoding either wild-type (wt) GFP-DGKα or a mutant in which Y335 was replaced by F (DGKαY335F). Analysis of pY in immunoprecipitated proteins showed that DGKα Y335F was phosphorylated to a much lower extent than the wt protein, suggesting that Y335 is a target for Lck-dependent phosphorylation (Fig. 2*B*), albeit not the only one. In contrast to results for the wt DGKα (29), enzymatic activity of the DGKαY335F mutant was not increased by cotransfection with Lck (Fig. 2*C*).

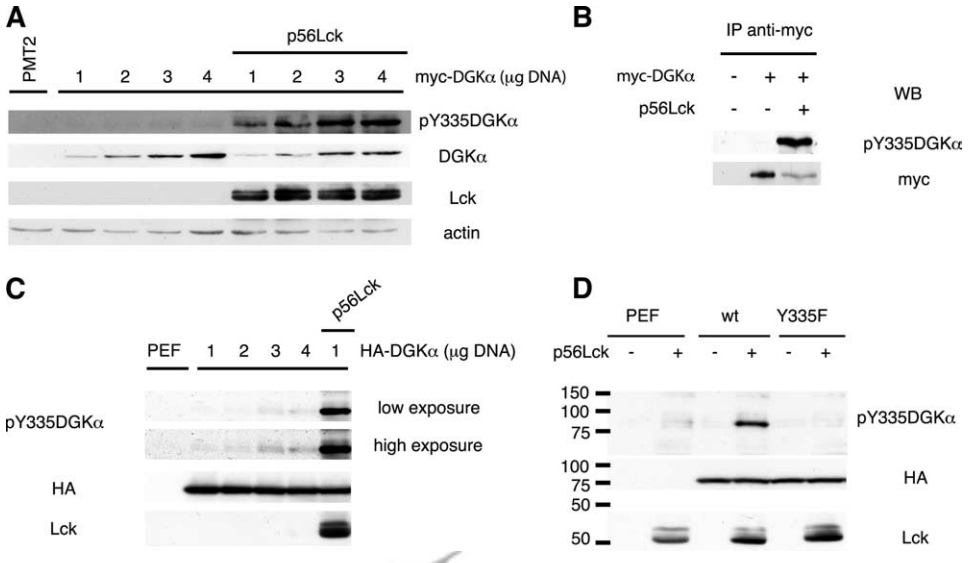

FIGURE 5. pY335 DGKα Ab recognizes DGKα tyrosine phosphorylation at Y335. *A*, HEK293 cells were transfected with different amounts of plasmid encoding *myc*-tagged human DGKα or empty vector (PMT2), alone or with a plasmid encoding Lck, as indicated. After 24 h, cells were collected and lysed, and pDGKαY335, DGKα, Lck, and actin levels were evaluated using the indicated Ab. *B*, *myc*-DGKα was immunoprecipitated, and blots were probed with anti-pDGK Y335. *C*, Same as in *A*, except that murine HA-tagged DGKα was used. *D*, HEK293 cells were transfected with a plasmid encoding HA-DGKα or HA-DGKα Y335F mutant, alone or with Lck. At 24 h posttransfection, cells were lysed, and proteins were resolved by SDS-PAGE and analyzed by Western blot with the indicated Ab.

Y335 determines DGKα function

These results prompted us to analyze whether impairment of DGKα Y335 phosphorylation affected enzyme translocation and/or function. Our previous studies established that DGKα membrane translocation is a rapid, transient event. Membrane-bound DGKα can be visualized in conditions that strongly inhibit enzyme relocalization, i.e., in the presence of DGK or tyrosine phosphatase inhibitors (27). We examined whether differences between TCR-triggered membrane localization of wt DGKα and the Y335F mutant could be detected by confocal analysis of live T cells. Jurkat cells were transfected with GFP-fused wt or Y335 mutant DGKα, and cells were plated on anti-CD3/CD28 mAb-coated plates, alone or with orthovanadate. In the case of the wt enzyme, membrane localization was observed (Fig. 3*A*, *left*); this was more pronounced when cells were pretreated with orthovanadate (data not shown). On the contrary, Y335F mutant-transfected cells did not show this membrane pattern (Fig. 3*A*, *right*), even in the presence of orthovanadate (data not shown). These results suggest that DGKα phosphorylation at Y335 is a decisive event for membrane stabilization of the enzyme.

Jurkat T cells express a large amount of endogenous DGKα, and enzyme overexpression does not affect TCR-dependent functions (27). We thus examined wt and mutated DGKα function in the HEK293 cell line, which expresses very low DGKα levels. PMA addition to HEK293 cells induced strong ERK phosphorylation (Fig. 3*B*), confirming that DAG-dependent signals regulate this mechanism (39). Expression of wt DGKα markedly reduced ERK phosphorylation, whereas the effect of expressing the nonphosphorylatable Y335F mutant was much less pronounced (Fig. 3*C*). This result confirms the role of DGKα as a negative modulator of DAG-dependent functions and demonstrates that phosphorylation of DGKα at Y335 is essential for enzyme function.

We have previously shown that lack of enzyme activity alters DGKα translocation kinetics, conferring dominant-negative properties on this kinase-dead DGKα mutant (DGKα KD). Expression of this mutant in Jurkat cells enhanced CD69 expression, as a consequence of promoted Ras/MAPK signaling (27). Because lack of activity does not prevent tyrosine phosphorylation of the enzyme (see Fig. 1*C*), we compared membrane translocation of DGKα KD with that of a construct bearing the Y335F mutation (DGKα KDY335F). Like its active counterpart, DGKα KDY335F failed to relocate to the membrane after stimulation (Fig. 4*A*). Accordingly, CD69 expression analysis indicates that Y335F mutation fully impaired the effect of the DGKα KD mutant (Fig. 4*A*). These results further support that Y335 phosphorylation is absolutely necessary for DGKα function at the membrane.

Generation of a phosphorylated Y335-specific Ab

To improve our analysis of DGKα phosphorylation at Y335, we generated a polyclonal phospho-specific Ab (pY335) and tested its specificity in in vitro studies using the wt and mutant versions of DGKα. We cotransfected HEK293 cells with a plasmid that coded for Lck and with increasing amounts of a plasmid encoding Myc-fused human DGKα. In these conditions, the pY335 Ab recognized human DGKα. Recognition was linear (Fig. 5*A*), and was further enriched following immunoprecipitation of DGKα (Fig. 5*B*). The pY335 Ab also recognized HA-tagged murine DGKα when overexpressed with Lck in HEK293 cells (Fig. 5*C*). Although the Ab clearly recognized DGKα when the cells coexpressed Lck, a weak signal was also observed in the absence of Lck (Fig. 5*C*, longer exposure). Because HEK293 cells express other PTK of the Src family, these kinases might phosphorylate the overexpressed DGKα.

To confirm the specificity of the pY335 Ab, we transiently transfected HEK293 cells with wt HA-tagged murine DGKα or DGKαY335F alone or together with a plasmid encoding Lck. Analysis of total cell lysates showed strong pY335 Ab reactivity with wt DGKα when cotransfected with Lck. The pY335 Ab did not recognize the DGKαY335F mutant, although wt and mutant proteins were expressed at similar levels (Fig. 5*D*).

To test endogenous DGKα phosphorylation on Y335 in Jurkat T cells, we analyzed total cell lysates in immunoblot using the pY335 Ab, which revealed a clear band corresponding to the M_r of DGKα (Fig. 6*A*). We used the Src family inhibitor PP2 to corroborate our hypothesis that DGKα phosphorylation was Src family kinase activity dependent. Analysis showed that DGKα phosphorylation at Y335 decreased at the same inhibitor dose that reduced tyrosine phosphorylation in total cell lysates (Fig. 6*A*). To examine the role of Lck in Y335 phosphorylation, we used JCaM, a Jurkat cell variant that lacks Lck (31). Although endogenous DGKα expression is similar in both cell lines, the pY335 Ab reacted strongly with endogenous DGKα in Jurkat, but not in JCaM cells (Fig. 6*B*). We also observed marked reactivity in the Molt4 leukemia T cell

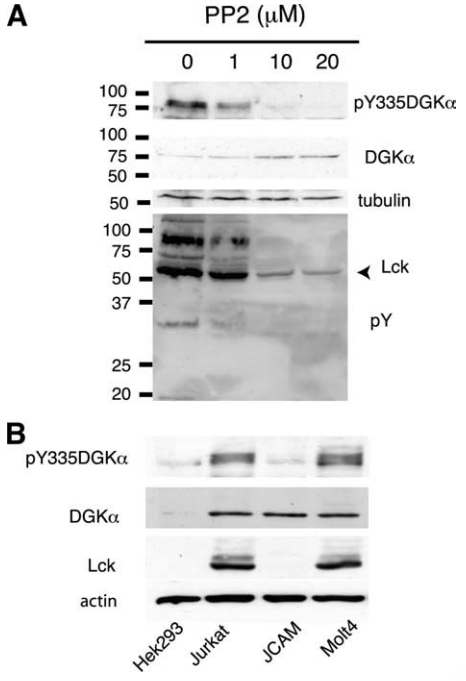

FIGURE 6. *A*, DGKα phosphorylation depends on a Src kinase activity. Jurkat cells were left untreated or incubated with the Src inhibitor PP2 at the indicated concentrations (1 h). Cells were collected and lysed, and proteins were resolved by SDS-PAGE and immunoblot using pY335 Ab to detect DGKα phosphorylation and anti-DGKα Ab to determine DGKα expression. *B*, DGKα phosphorylation depends on Lck expression. Total cell lysates from HEK293, Jurkat, JcaM, and Molt4 cells were analyzed by SDS-PAGE. Blots were probed with pY335 Ab to detect DGKα phosphorylation. DGKα expression and Lck expression were determined using specific DGKα and Lck Abs.

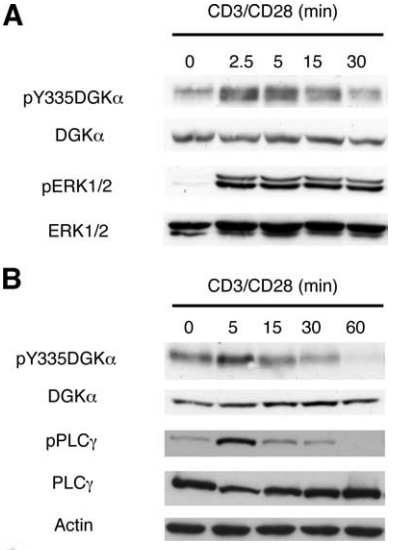

FIGURE 7. TCR stimulation increases DGKα Y335 phosphorylation. *A*, Jurkat cells were stimulated with anti-CD3 and anti-CD28 for the times indicated. Cells were lysed and proteins were analyzed by SDS-PAGE and immunoblot. Nitrocellulose membranes were blotted with the indicated Ab. *B*, As in *A*, but membranes were blotted with anti-pDGKα-Y335 and anti-pPLCγ Y783 and reprobed with anti-DGKα, anti-PLCγ, and anti-actin to detect total proteins.

line, which also expresses Lck. These experiments confirm phosphorylation of endogenous DGKα at Y335 and suggest that, in T lymphocytes, this phosphorylation is Lck dependent.

TCR triggering induces DGKα phosphorylation at Y335

We showed above that DGKα tyrosine phosphorylation is Lck dependent. TCR triggering activates Lck and also generates DAG and an increase in $[Ca^{2+}]i$. We next determined DGKα Y335 phosphorylation kinetics following TCR triggering. The basal Y335 phosphorylation observed in Jurkat T cells increased rapidly following TCR triggering, to then decrease at longer times (Fig. 7*A*). These rapid, transient kinetics mirrored those of PLCγ, suggesting a correlation between the mechanisms that govern DAG generation and consumption (Fig. 7*B*).

Effect of costimulation on DGKα Y335 phosphorylation

DGKα tyrosine phosphorylation is observed in the absence of the CD28 costimulatory signal (Fig. 1, *A* and *B*). To evaluate the importance of costimulation in DGKα Y335 phosphorylation kinetics, we stimulated Jurkat cells with anti-CD3, alone or with anti-CD28 Ab. We observed phosphorylation even after serum deprivation, when the total tyrosine phosphorylation activity level is greatly decreased. Anti-CD3 stimulation was sufficient to induce robust DGKα Y335 phosphorylation (Fig. 7); after costimulation, phosphorylation was also observed, albeit with more transient kinetics than following anti-CD3 stimulation (Fig. 8*A*).

We next determined whether DGKα was also phosphorylated in response to TCR ligation in human primary T cells. We detected DGKα phosphorylation in unstimulated PBL, which increased further following TCR triggering. As also found for Jurkat T cells, phosphorylation decreased more rapidly when cells were costimulated (Fig. 8*B*).

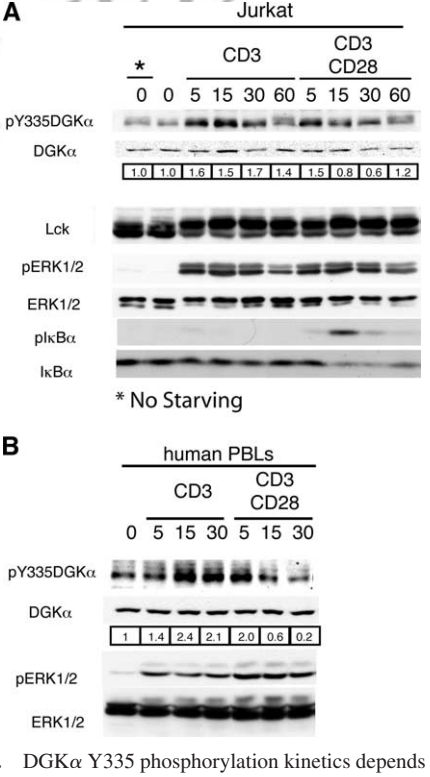

FIGURE 8. DGKα Y335 phosphorylation kinetics depends on costimulatory signals. *A*, Jurkat cells were stimulated with anti-CD3 alone or anti-CD3/CD28 for the times indicated and lysed, and proteins were resolved by SDS-PAGE and analyzed by immunoblot with anti-pDGKα Y335. Membranes were then reblotted with anti-DGKα to detect total protein. Immunoblots of duplicate samples were probed with anti-Lck, anti-pERK1/2, and anti-pIκB as a control of stimulation. DGKα phosphorylation was quantified with Image J software. *B*, Human PBLs were purified, as indicated in *Materials and Methods*, and treated as in *A*.

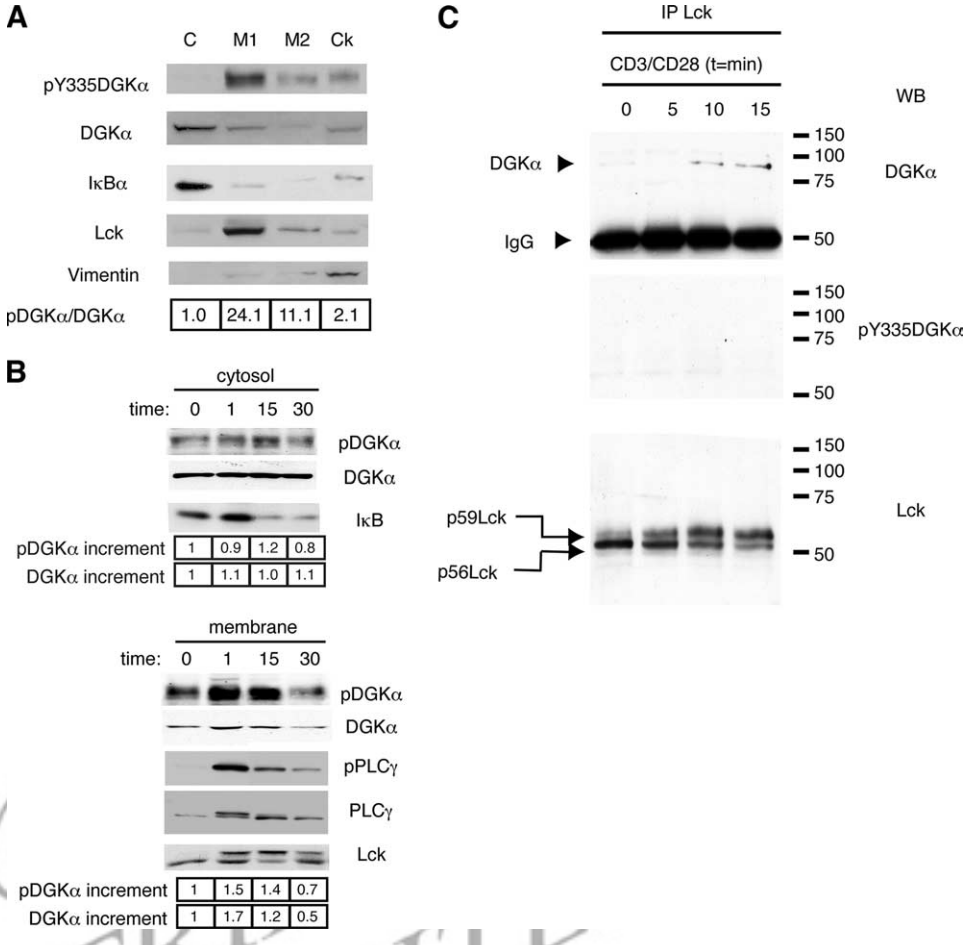

FIGURE 9. *A*, DGKα pY335 DGKα subcellular distribution. *A*, Jurkat subcellular fractions were isolated by fractionation, as described in *Materials and Methods*. The proteins obtained in the sequential extractions were resolved by SDS-PAGE, and Western blot membranes were blotted with anti-Lck, anti-IκBα, and anti-vimentin Abs as membrane, cytosol, and cytoskeleton isolation controls, respectively. Anti-pDGKα-Y335 and anti-DGKα were used to analyze phosphorylated DGKα and DGKα cellular distribution. *B*, Jurkat cells were stimulated by TCR cross-linking with anti-CD3/CD28, and DGKα phosphorylation was assessed in the cytosol and membrane fractions, as in *A*. *C*, DGKα associates to Lck in response to TCR stimulation. Lck was immunoprecipitated from lysates of Jurkat cells stimulated for the indicated times with anti-CD3/CD28. Total cell lysates and immunoprecipitated pellets were analyzed by SDS-PAGE and immunoblot, and membranes were blotted with the indicated Ab.

DGKα phosphorylated at Y335 represents a membrane-bound DGKα pool

In Jurkat T cells, we previously showed rapid, transient translocation to membranes of both GFP-DGKα fusion proteins and the endogenous enzyme in response to receptor stimulation (24, 27) (Fig. 3*A*). Analysis of endogenous DGKα by subcellular fractionation of murine lymph node T cells shortly after in vivo engagement of the TCR confirmed that this enzyme, which is cytosolic in resting T cells, translocated to the membrane in response to receptor triggering (24). To analyze the correlation between DGKα Y335 phosphorylation and membrane location, we determined the location of phosphorylated DGKα in subcellular fractions of Jurkat T cells. We found that DGKα is present mainly in the cytosolic fraction, with a very small amount in the membrane fraction, as previously observed (24). The pY335 Ab did not recognize DGKα in cytosol, but only in membrane fractions, where Lck is also located (Fig. 9*A*). This demonstrates that phosphorylation at Y335 is only detected when DGKα is located at the membrane, and suggests that the pY335 Ab represents an excellent tool for detection of the membrane-associated/active DGKα fraction.

Because phosphorylated DGKα appears to represent the membrane-bound pool, we tested phosphorylation kinetics in membranes isolated after stimulation. We identified changes in phosphorylation almost exclusively in the membrane fraction, whereas phosphorylation of the cytosolic protein was barely detected (Fig. 9*B*).

The finding that DGKα Y335 phosphorylation was Lck activity dependent and that, like Lck, DGKα pY335 was found exclusively in the membrane fraction, suggested that DGKα translocation to the membrane is required for Lck-dependent phosphorylation. We thus analyzed the possible association between these two proteins. We stimulated Jurkat cells with anti-CD3/CD28 mAb, followed by Lck immunoprecipitation and DGKα detection in the complexes. Following stimulation, we detected DGKα in anti-Lck pellets (Fig. 9*C*). The pY335 Ab nonetheless indicated that the Lck-associated DGKα fraction was not phosphorylated at this residue. These data suggest that, whereas Lck associates DGKα, tyrosine phosphorylation at residue 335 induces DGKα dissociation from the complex while maintaining the enzyme at the membrane. This indicates that TCR-dependent Lck activation facilitates interaction with DGKα, allowing phosphorylation at Y335, which in turn promotes DGKα stabilization at the membrane, while causing its dissociation from Lck.

Ca^{2+} flux enhances DGKα phosphorylation at Y335

DGKα has two EF hand domains, characteristic of the Ca^{2+}-binding proteins, and is activated by Ca^{2+} in vitro (23). Deletion of the EF hand domains induces constitutive membrane localization, suggesting that a $[Ca^{2+}]$i-dependent conformational change is necessary to allow membrane localization of the enzyme (27). We therefore evaluated the individual effects of $[Ca^{2+}]$i and DAG on DGKα phosphorylation at Y335. We raised $[Ca^{2+}]$i levels using the calcium ionophore ionomycin; to mimic DAG generation, we used PMA, which is often used as a costimulatory signal in TCR stimulation (40, 41). Ionomycin treatment promoted a considerable increase in DGKα Y335 phosphorylation, as well as NF-AT translocation to the nucleus (Fig. 10). Although PMA addition strongly induced ERK phosphorylation, it did not affect Y335 phosphorylation. Finally, maximum Y335 phosphorylation was observed using PMA and ionomycin together (Fig. 10*A*). These

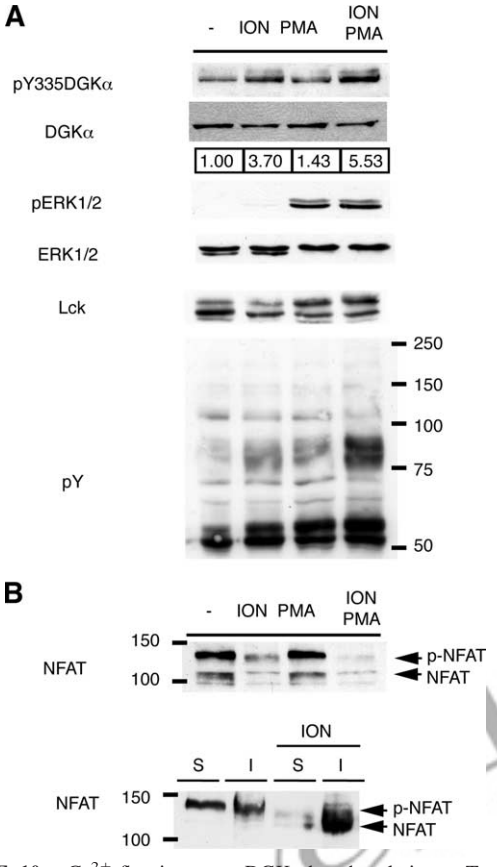

FIGURE 10. Ca^{2+} flux increases DGK phosphorylation at Tyr^{335}. Jurkat cells were left untreated or stimulated with Ca^{2+} ionophore (ionomycin), PMA, or both (5 min). Cells were lysed, and proteins were separated by SDS-PAGE. *A*, Nitrocellulose membranes were blotted with anti-pY335DGKα and anti-pERK1/2 Ab as a control of PMA stimulation, and anti-DGKα, anti-ERK1/2, and anti-Lck Ab as protein controls. Phosphorylated proteins were detected with anti-pY mAb. *B*, Duplicate samples were probed with anti-NF-AT Ab to visualize NF-AT dephosphorylation (*top*) and translocation to the insoluble fraction (*bottom*).

results demonstrate that the concerted activation of DAG and Ca^{2+}-dependent signals induces maximum DGKα phosphorylation at Y335. Ca^{2+} mobilization alone, probably through direct binding to the EF hand motifs, appears to induce a conformational change that facilitates enzyme phosphorylation.

Discussion

The exquisite tuning of the TCR is possible because this receptor is coupled to signaling networks controlled by down-regulator proteins and interconnected negative feedback loops. TCR activation initiates a cascade of tyrosine phosphorylation that leads to DAG production and Ca^{2+} release. Tyrosine phosphorylation is controlled and terminated by the action of different tyrosine phosphatases (33), such as Src homology (SH) region 2 domain-containing phosphatase-1 (SHP-1), whereas DAG levels are strictly controlled by DGK action (16, 17, 24).

The Src kinase Lck is one of the main PTK activated during TCR triggering. In addition to its enzymatic function, this kinase serves as an adaptor protein through its SH domains, SH2 and SH3 (7). Recent studies using naive T cells from Lck-deficient mice pointed out that through Y136 LAT phosphorylation, Lck is the main trigger for PLCγ activation. Lck is thus the main Src kinase controlling subsequent DAG production and activation of DAG-responsive molecules, such as Ras-GRP1 and PKCθ (8). Lck also exerts a suppressive role on the TCR, either through TCR internalization (42) or by turning on a negative feedback loop through SHP-1 (SH2-containing PTP type I) activation. Lck phosphorylates SHP-1 on residues Y536 and Y566, leading to an increase in SHP-1 phosphatase activity. Lck down-regulation by small interfering RNA in Jurkat cells thus suppresses proximal TCR signaling and also increases the downstream response, evaluated as ERK phosphorylation and IL-2 production (43). AQ: J

In this study, we demonstrate that Lck also phosphorylates another negative regulatory molecule of the TCR response, DGKα. Consequently, Lck controls not only the switching off of tyrosine phosphorylation, but also the termination of DAG-derived signals. Phosphorylation of DGKα by a PTK activated during TCR triggering concurs with our previous results establishing that in addition to Ca^{2+} flux, DGKα membrane translocation requires tyrosine phosphorylation (27).

We identified Y335, located at the hinge between the C1 and the catalytic domains, as a Lck-dependent DGKα phosphorylation site. Generation of a specific Y335DGKα Ab demonstrated that, both in T cell lines and primary human lymphocytes, DGKα is phosphorylated at this residue. Phosphorylation of endogenous DGKα is inhibited by Src-family PTK inhibitors. Experiments comparing Jurkat T cells and the Lck-deficient variant indicate that, at least in T lymphocytes, Lck is the PTK responsible for DGKα phosphorylation.

Other Src family PTK (Src, Fyn, Lyn, Blk, Yes, Fgr, Hck) may be able to phosphorylate DGKα at this residue in nonlymphoid cells. In HEK293 cell overexpression experiments, we detected weak Y335 phosphorylation of DGKα, even in the absence of Lck. HEK293 cells do not express Lck, although they express other Src kinases that might phosphorylate the ectopically expressed DGKα. Accordingly, Src kinases are reported to be essential for DGKα modulation in various adherent cell lines in response to receptors such as VEGF, HGF, or α-D-tocopherol (30, 37, 38, 44). AQ: K

Experiments with overexpressed enzyme suggest that DGKα Y335 phosphorylation targets DGKα to the plasma membrane (37, 38). Analysis of endogenous protein in Jurkat T lymphocytes demonstrates that tyrosine-phosphorylated DGKα represents a membrane-associated fraction, which probably corresponds to the active pool of the enzyme. This is consistent with the fact that the small amount of phosphorylated DGKα, derived from DGK overexpression in HEK293 cells, reduces ERK phosphorylation. In addition, impairment of Y335 phosphorylation in DGKα KD dramatically inhibits the dominant-negative properties of this mutant, further suggesting a direct correlation between Y335 phosphorylation and DGKα function.

During TCR activation, tyrosine phosphorylation of DGKα (as detected with total pY or pY335DGKα Ab) increases to a maximum level, which then decreases sharply. This pattern resembles the tyrosine phosphorylation kinetics of PLCγ, suggesting that Lck activation turns on a coordinated mechanism that controls DAG production and consumption in response to TCR (Figs. 7 and 11). F11 There may be similar coordination in the modulation of PLCβ-generated DAG, because DGKα modulates DAG produced by PLCβ following activation of the carbachol receptor (27). DGKα and Lck participation in the signaling by other GPCR, such as the chemokine receptors, remains to be determined. AQ: L

Because DGKα requires both tyrosine phosphorylation and Ca^{2+} for full activation, it is reasonable to assume that DGKα is phosphorylated only after a $[Ca^{2+}]i$ increase; this idea is also supported by our experiments using PMA and the Ca^{2+} ionophore ionomycin. Although Ca^{2+} influx increases DGKα Y335 phosphorylation, PMA alone does not. This shows that Ca^{2+} enhances Lck-dependent DGKα phosphorylation, and further supports a

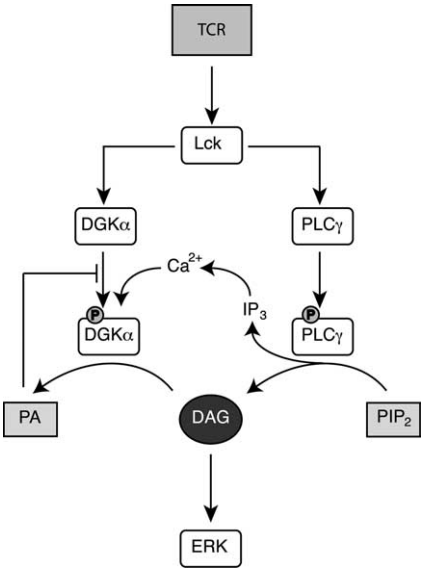

FIGURE 11. Model of TCR-mediated DGKα activation. Following TCR engagement, Lck is activated and triggers a signaling cascade that leads to PLCγ activation, which generates DAG and IP_3. This last messenger provokes a Ca^{2+} flux from endoplasmic reticulum that promotes a conformational change in the DGKα. In its open conformation, DGKα is able to interact with Lck at the plasma membrane. This interaction leads to DGKα phosphorylation at Y335, which induces its stabilization at the membrane and its rapid dissociation from Lck. The pY335 DGKα is active and, because it is membrane bound, can easily metabolize the TCR-generated DAG. DGKα activity at the membrane down-regulates DAG-dependent signals and at the same time promotes its own inactivation, most likely through a PA-sensitive tyrosine phosphatase.

model in which Ca^{2+} influx induces a closed-to-open conformation transition of DGKα, allowing its interaction with membrane (27).

Lck-dependent DGKα phosphorylation suggests a direct interaction between these two proteins (45). Our fractionation experiments indicate that DGKα pY335 is located at the membrane, where Lck is also concentrated, and TCR stimulation induces an increase in the phosphorylation of the protein at the membrane. Rapid association of DGKα and Lck is observed after TCR triggering, concurring with earlier studies reporting an association between DGKα and Src kinase activity (30, 44, 46). It is striking that DGKα pY335 is not observed in the Lck immunoprecipitates. Although we cannot discard resolution limitations of the pY335 Ab, this would appear to indicate that phosphorylation of DGKα induces its rapid dissociation from Lck, simultaneously allowing DGKα association with the plasma membrane. The physiological significance of this rapid loss of association remains to be determined, but it may favor DGKα association with other partners. A recent publication showed that phosphorylated Y335 might interact with certain SH2 domains in vitro (37). The rapid dissociation of DGKα pY335 and Lck might also control and/or restrict an inhibitory DGKα effect on Lck activity. ERK phosphorylates Lck, converting the $p56^{Lck}$ isoform to the phosphorylated $p59^{Lck}$ form. This phosphorylation affects the SH2 tyrosine-binding properties of Lck (47), primarily its binding and subsequent inactivation by SHP-1 (48). Transitory activation of DGKα, through its effects on Ras/ERK pathway, may indirectly influence the temporality of Lck activity.

Because DAG metabolism is essential during TCR-mediated responses, it is important to understand the mechanisms that control DGKα activation. Our results demonstrating DGKα tyrosine phosphorylation at Y335 in response to TCR ligation in cultured cell lines and in primary human PBLs reveal a new aspect of DGKα regulation. It is interesting that CD28-mediated costimulation causes a more transient phosphorylation of Y335, suggesting that costimulation results in a more rapid termination of DGKα-mediated signals. Although the mechanism by which CD28 orchestrates the temporality of DGKα activation remains unknown, DGKα inactivation by costimulatory signals correlates with the proposed role of this DGK isoform as a negative regulator of T cell functions and of an anergy-induced gene (14). We previously showed that DGKα overexpression prevents Ras-GRP1 membrane localization (24), thereby blocking the Ras/ERK pathway. Recent studies of DGKα overexpression in an in vivo model of T lymphocyte activation confirm these results, and demonstrate that DGKα contributes to establishment of anergy (49). This function concurs with studies using DGKα-deficient mice, in which the absence of DGKα contributes to an anergy-resistant state, and indicates that DAG metabolism is essential to anergy development (17).

TCR engagement with its ligand leads to a cellular response in which different factors are integrated, to give rise to either activation or tolerance. In the periphery, low-affinity ligands such as self-ligands lead to an inefficient response and thus to tolerance; in contrast, pathogenic ligands elicit strong, productive activation that promotes effective host defense. Our finding suggests that those stimuli that provoke tolerance or anergy could induce disproportionate DGKα activation, either through Ca^{2+} flux or initial Lck activity. In such cases, loss of temporality of the DGKα response would block the Ras/ERK pathway and, through SHP-1, turn off Lck, leading to incomplete, nonproductive T cell activation. Moreover, tolerized T and B lymphocytes show a basal increase in $[Ca^{2+}]i$ levels (14, 50). It is not known whether such control mechanisms are applicable to other DGK isoforms. At difference from DGKα, DGKζ activity does not appear to be regulated directly by Ca^{2+} flux, although this isoform is reported to associate with a Src kinase activity in gonadotrophic cells (51).

In summary, our data suggest a model in which TCR-dependent activation of Lck regulates DAG generation and removal through the concerted activation of PLCγ and DGKα (Fig. 11). Tyrosine phosphorylation of DGKα is facilitated by the PLCγ-generated Ca^{2+} flux. This Ca^{2+}-dependent priming of DGKα provides a unique mechanism that guarantees the correct timing of PLCγ and DGKα activation.

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Disclosures

The authors have no financial conflict of interest.

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