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*J Immunol* published online 2 November 2011
http://www.jimmunol.org/content/early/2011/11/02/jimmunol.1002476

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Supplementary Material

http://www.jimmunol.org/content/suppl/2011/11/02/jimmunol.1002476.DC1

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SAP-Mediated Inhibition of Diacylglycerol Kinase α Regulates TCR-Induced Diacylglycerol Signaling

Gianluca Baldanzi,*1 Andrea Pighini,*1 Valentina Bettio,* Elena Rainero,*2 Sara Traini,* Federica Chianale,* Paolo E. Porporato,*3 Nicoletta Filigheddu,* Riccardo Mesturini,† Shuping Song,† Tamás Schweighoffer,§ Laura Patrucci,§ Cosima T. Baldari,§ Xiao-Ping Zhong,‖§, Wim J. van Blitterswijk,**, Fabiola Sinigaglia,* Kim E. Nichols,†† Ignacio Rubio,‡ Ornella Parolini,‡‡ and Andrea Graziani*

Diacylglycerol kinases (DGKs) metabolize diacylglycerol to phosphatidic acid. In T lymphocytes, DGKα acts as a negative regulator of TCR signaling by decreasing diacylglycerol levels and inducing anergy. In this study, we show that overexpression of DGKα in T cells, engaged by specific Ags, along with stimulation by costimulatory receptors such as CD28, leads to T cell activation, cytokine production, and differentiation. Moreover, several other receptors influence cell activation by quantitatively or qualitatively modifying immunoreceptor-derived signals. Conversely, stimulation via the TCR alone, although partially activating intracellular signaling pathways, is not sufficient to induce effector functions such as cytokine production and proliferation (1).

Signaling lymphocyte activation molecule (SLAM; CD150) is a homotypic transmembrane receptor expressed in T and B lymphocytes, dendritic cells, and monocytes (2). Upon engagement, SLAM undergoes a conformational change leading to Fyn-mediated tyrosine phosphorylation and activation of several signaling pathways that modulate TCR-induced responses (2). Fyn recruitment to the activated SLAM is mediated by SAP, an adaptor protein comprising a single SH2 domain and a SH3 domain-binding sequence (3). In humans, SAP loss-of-function mutations cause X-linked lymphoproliferative disease (XLP), an immune disorder characterized by a deregulated immune response to EBV, susceptibility to lymphoma and defective Ab production (4). Interestingly, SAP-deficient T lymphocytes from either XLP patients or SAP knockout mice exhibit defective responses to TCR/CD28 costimulation in vitro: 1) T cells from XLP patients feature reduced ERK1/2 and NF-κB activation, decreased IL-2 production, and impaired proliferation (5); 2) CD4+ T cells from XLP patients exhibit reduced ICOS expression and IL-10 production (6); and 3) T cells from SAP knockout mice feature reduced protein kinase C (PKC) membrane recruitment, Bcl-2 phosphorylation, and NF-κB activation, which are associated with defective IL-4 secretion and enhanced INF-γ production (7).

Ag-mediated activation of the TCR in the presence of other co-activating molecules triggers a complex signaling network leading to...
to transcriptional activation of specific genes whose expression mediates T cell proliferation and differentiation. Activation of Ras and PKC0 triggers key signaling pathways, leading, among others, to the activation of NF-AT and NF-κB and contributing to transcription of the IL-2 gene (8, 9). In T cells, activation of Ras and PKC0 is dependent on the generation of diacylglycerol (DAG) through phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bis-phosphate. DAG recruits RasGRP, the Ras-GEF mainly responsible for TCR-induced Ras activation, and PKC0 to the plasma membrane (10, 11). Notably, engagement of TCR in the absence of costimulation results in a weak and transient activation of both Ras and PKC0, which drives T cells into anergy, a hypertensive status characterized by the inability to produce IL-2 and proliferate (12, 13).

DAG generated upon T cell activation is rapidly metabolized by DAG kinases (DGKs), a multigenic family of enzymes responsible for phosphorylation of DAG to phosphatidic acid (PA). Consistently with the crucial role of DAG signaling in T cell activation, several pieces of evidence indicate that the DGKα and DGKζ isoforms, which are highly expressed in thymus and T cells, act as negative regulators of TCR signaling and immune cell function (14). Specifically, 1) genetic deletion of DGKα and DGKζ in T cells enhances TCR-induced activation of ERK1/2, resulting in defective induction of anergy (15, 16); 2) DGKα is strongly induced in anergic T cells (13); 3) overexpression of either DGKα or DGKζ impairs CD3/CD28-induced activation of Ras signaling (17–19); 4) pharmacological inhibition of DGKα reverses the inability of anergic cells to produce IL-2 in response to TCR stimulation (13); and 5) DGKα expression is downregulated within a few hours from T cell activation (19). Collectively, these data support the concept that second messengers signaling is highly dependent on the fine tuning of DAG synthesis and degradation rates. Although there is no evidence for regulation of DGKζ upon T cell activation, TCR/CD28 costimulation of T cells results in rapid and sustained recruitment of DGKα to the plasma membrane (19), an event mediated by both Lck-dependent phosphorylation of tyrosine 335 and calcium binding to the EF hand domain of DGKα (20, 21).

Based on the role of DGKα as a negative regulator of T cell responses, we investigated the hypothesis that, upon T cell stimulation, DGKα activity might undergo negative regulation. In this study, we show indeed that the enzymatic activity of DGKα is inhibited upon costimulation of TCR and CD28 through a SAP-mediated mechanism. Moreover, we found that, in SAP-deficient cells, defective TCR/CD28 signaling and T cell activation can be partially rescued by inhibition of DGKα.

Materials and Methods

Cell culture

Jurkat A3 cells (LCG Standards) and 293FT cells (Life Technologies) were cultured as described in RPMI 1640 GlutaMAX medium or DMEM GlutaMAX high glucose (Life Technologies), supplemented with 10% FBS (Life Technologies) and antibiotic-antimycotic solution (Sigma-Aldrich) in humidified atmosphere with 5% CO₂. For immunoprecipitation, 3 x 10⁷ cells were lysed in 1 ml lysis buffer A (25 mM HEPES [pH 8], 1% Nonidet P-40, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM ZnCl₂, 50 mM ammonium molybdate, 10 mM NaF, 1 mM sodium orthovanadate, and protease inhibitor mixture from Sigma-Aldrich). An aliquot of cell lysate was retained for Western blot analysis, and the remainder was immunoprecipitated with a mixture of anti-DGKα Abs as previously described (24). Whole-cell homogenates were prepared by homogenizing 3 x 10⁷ cells in 1 ml cold buffer B (buffer A without detergent) by 20 passages in a 23-gauge syringe. Protein concentration was determined by BCA (Pierce), and equal amounts of proteins were loaded in each lane. SDS-PAGE and Western blots were performed as described previously (25). Western blot results were acquired with a VersaDoc system and quantified using Quantity One software (Bio-Rad).

Expression vectors and transfections

GFP-SAP-wild type, GFP-SAP-R78A, and GFP-SAP-R55L were a gift of J. Vavillette (Montreal, QC, Canada). Jurkat/SAP-shRNA cells were obtained by infection with lentiviruses encoding a shRNA specific for murine DGKα in pLKO.1-Puro vector (clone ID TRCN000000 82712 RNAi Consortium through Sigma-Genosys), sequence: 5'-CCGGCACAGGTTACTACGAGGATA-CTCCGGAGTACCCGATTTGTTTTG-3'. Jurkat/control-shRNA cells were obtained by infection with lentiviruses encoding a shRNA specific for murine DGKα in pLKO.1-Puro vector (clone ID TRCN000000 24825 R4N1 Consortium through Sigma-Genosys), sequence: 5'-CCGGAGACTTAAAGGTTGGATATCCTGAGATATAC-CACCTTACCTTACGCTTTT-3'. Lentivirus production and Jurkat infection were carried out according to the manufacturer’s instructions. Infected Jurkat cells were selected for 14 d in purinomycin (1 μg/ml) and used as a bulk population in all experiments.

Reagents

The Abs used recognize the following proteins: pan-Ras (Ab-4; Merck), H-Ras (F235; Cell Signaling Technology), linker for activation of T cells (LAT; Santa Cruz Biotechnology), anti–IL-2α receptor Ab (TAC; Abcam), CD3 antigen (OKT3; provided by U. Dianzani, Novara, Italy), CD28 agonist (ANC28.1/5D10; Ancell) (except for Fig. 4D, where anti-CD28 was from BD Pharmingen), SLAM agonistic Ab (A12; BioLegend), anti-DGK Abs (gift of M. Topham, Salt Lake City, UT), mixture of DGK Abs used for immunoprecipitation (22), DGKα (C-20) and PKCθ (Santa Cruz Biotechnology) used for immunofluorescence, ERK1/2 and phospho-ERK1/2 from Cell Signaling Technology for Supplemental Fig. 2 and from Transduction Laboratories for Fig. 5C; SAP (FL-128; Upstate Biotechnology), α-tubulin (Sigma-Aldrich), secondary HRP-conjugated Abs (PerkinElmer), secondary FITC-conjugated Ab (Dako), and Alexa Fluor 546-phalloidin (Life Technologies). In all experiments involving stimulation with Abs, species-matched preimmune serum (Santa Cruz Biotechnology) was used for controls in equal amounts.

Inhibitors used were from Sigma-Aldrich: R59922, DGKα inhibitor; PP2, Src family inhibitor; U73122, PLC inhibitor; BA-PAT-AM, cell-permeable calcium chelator; wortmannin, PI3Ks inhibitor; and IPA-3, PAK-specific inhibitor. BA-PAT-AM was dissolved in water; others inhibitors were dissolved in DMSO. DMSO was always used in control samples at the same dilution as the inhibitor tested.

Expression vectors and transfections

GF-P-SAP-wild type, GF-P-SAP-R78A, and GF-P-SAP-R55L were a gift of P. Schwartzberg (National Institutes of Health, Bethesda, MD). N-terminal yellow fluorescent protein (YFP)-DGKα was obtained by cloning DGKα in YFP-P-DIEST (Life Technologies) using the Gateway kit (Life Technologies) according to the manufacturer’s instructions, pN-F-AT-Luacerase reporter vector and pRL-TK normalization vector were from Clontech. Small interfering RNA (siRNA) and negative control siRNA were from Ambion/Life Technologies: DGKα siRNA (23) sense, 5'-GGUCA-GUGAGUGCUACAAAGTTT-3', antisense, 5'-CUUAGACAGUACUCAUGAC-3'. Transient transfections in Fig. 5D and 5E were performed using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer’s instructions. Microporation of Jurkat cells for imaging experiments was performed according to the manufacturer’s instructions with the Microporator MP-100 system from Digital Bio Technology (Fig. 2, Supplemental Fig. 3B) or with the Gene Pulser II from Bio-Rad (Fig. 5B).
**Immunofluorescence**

For immunofluorescence on fixed cells with Ab stimulation, cells were seeded on poly-l-lysine-coated glass coverslips (MARIENFELD) in 24-well plates for 1 h and then stimulated with 10 μg/ml agonist Abs for 1 h in the presence or absence of the indicated inhibitors. Cells were then fixed with formaldehyde and stained as previously described (26). Confocal images were acquired with a Leica confocal microscope TSP2 (objective, ×63; numerical aperture, 1.32) and analyzed with LCS confocal software (Leica).

For the immunological synapse experiments, Raji cells (used as APCs) were incubated for 2 h with 10 μg/ml staphylococcal enterotoxin E. Raji cells were washed, mixed with Jurkat control-shRNA or Jurkat SAP-shRNA (1:1) for 15 min, and plated on polylysine-coated wells of diagnostic microscope slides (Erie Scientific). Cells were allowed to adhere for 15 min and then fixed in methanol at −20°C for 10 min. Samples were then washed for 5 min in PBS and incubated with anti-PK-C9 Ab overnight at 4°C. After washing in PBS, samples were incubated for 1 h at room temperature with FITC-labeled anti-goat Ab. Images were taken using an Axio Imager Z1 microscope equipped with an HBO 50-W mercury lamp for epifluorescence and with an AxioCam HR cooled charge-coupled camera (Carl Zeiss).

For live cell imaging experiments, Jurkat A3 cells, Jurkat control-shRNA, and Jurkat SAP-shRNA were microporated and serum starved in RPMI 1640 supplemented with 0.2% BSA plus 50 mM HEPES for 2 h. Cells were seeded on glass-bottom dishes coated with poly-l-lysine or with the agonistic Ab anti-CD3, anti-CD3 plus anti-SLAM, or anti-CD3 plus anti-CD28 at the final concentration of 10 μg/ml. Confocal images were acquired at the indicated times with a Zeiss LSM 510 inverted laser scanning microscope using a C-Apochromat ×63 water immersion objective lens (Carl Zeiss). Laser scanning microscope image files were processed using the Zeiss ZEN laser scanning microscope image browser software. When comparisons among images were to be made, the images were taken in identical conditions and equally manipulated using Adobe Photoshop 7.0 software (Adobe Systems).

**Cell fractionation**

Cells (3 × 10^7/ml) were resuspended in RPMI 1640 and incubated for the indicated time with agonist Abs or control species-matched preimmune serum at 37°C. Whole-cell homogenates were prepared by homogenizing pRL-TK plasmids. After 48 h, cells were stimulated as indicated for 16 h, lysed in 1 ml ice-cold lysis buffer (50 mM HEPES [pH 7.5], 140 mM NaCl, 5 mM MgCl2, 1 mM DTT, 1% Nonidet-40, protease inhibitors) supplemented with 25 μg GST-RBD protein and 100 μM GDP to quench postlytic GTP-loading and GAP-dependent Ras-bound GTP hydrolysis, respectively. Cell extracts were cleared by centrifugation and GST-RBD/Ras-GTP complexes were collected on glutathione-Sepharose, washed once with lysis buffer, and processed for SDS-PAGE analysis.

**Biochemical Ras activation assays**

Recombinant GST–Raf-RBD protein was produced in *Escherichia coli* as described (27). Jurkat cells were serum deprived (2 h in RPMI 1640 supplemented with 0.2% fatty acid-free/endothoxin-low BSA and 50 mM HEPES [pH 7.5]). After stimulation, 1 ml cell suspension (10^7 cells) was lysed in 1 ml ice-cold lysis buffer (50 mM HEPES [pH 7.5], 140 mM NaCl, 5 mM MgCl2, 1 mM DTT, 1% Nonidet-40, protease inhibitors) supplemented with 25 μg GST-RBD protein and 100 μM GDP to quench postlytic GTP-loading and GAP-dependent Ras-bound GTP hydrolysis, respectively. Cell extracts were cleared by centrifugation and GST-RBD/Ras-GTP complexes were collected on glutathione-Sepharose, washed once with lysis buffer, and processed for SDS-PAGE analysis.

**Mammalian two hybrid system**

A modified Clontech MatchMaker (BD Biosciences) mammalian two-domain–hybrid assay was used. Full-length human SAP and its point-mutated variants were cloned into the pM series vectors as GAL4-binding domain (BAD) hybrids. For the immunological synapse experiments, either full-length DGKα or the N-terminal DGKα fragment was cloned into a pVP vector to direct expression of VP16-activation domain fusion proteins. These were cotransfected into subconfluent HEK293 cells with a GAL4-luciferase reporter plasmid and a pVAX-based expression plasmid containing full-length human FynT. Luciferase activity was measured after 24 h using a cell-based kit (Promega).

**NF-AT assay**

Jurkat cells (4 × 10^6/ml) were cotransfected with pNF-AT-TA-luc and pRL-TK plasmids. After 48 h, cells were stimulated as indicated for 16 h. Luciferase was assayed with a Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions and assayed using a Victor3 V multilabel counter (PerkinElmer). NF-AT–driven firefly luciferase activity was normalized for the reference Renilla luciferase activity to take in account differences in transfection and expression efficiency, and all values were expressed as fold increase upon unstimulated controls.

**IL-2 assay**

Jurkat cells (1 × 10^6) were plated in 100 μl medium supplemented with 10% FBS and stimulated as indicated for 72 h. IL-2 released in the media was measured by ELISA (GE Healthcare).

**Statistical analysis**

The data were expressed as means ± SE. Statistical analysis was determined by a Student *t* test.

**Results**

**Negative regulation of DGKα during T cell activation**

Because DGKα negatively regulates T cell activation (17, 19), we set out to investigate whether it is regulated in the early phase of lymphocyte activation. To this purpose, we assayed the enzymatic activity and subcellular localization of DGKα upon activation of primary lymphocytes (PBLs) and Jurkat leukemic T cells. DGKα activity was measured in vitro in the presence of exogenous substrates in anti-DGKα immunoprecipitates obtained from either control or stimulated lymphocytes. Following 15 min costimulation of PBLs with agonistic anti-CD3 and anti-CD28 Abs, the enzymatic activity of DGKα was reduced by ∼60% as compared with unstimulated cells (Fig. 1A), without any change in DGKα protein content (Fig. 1A, lower right panel). Stimulation of PBLs with anti-CD3 Ab alone did not significantly affect DGKα activity (data not shown). Because activation of SLAM family receptors was reported to enhance TCR signaling (28, 29), we investigated whether SLAM might regulate the enzymatic activity of DGKα. Indeed, 15 min costimulation of PBLs with anti-CD3 and anti-SLAM agonist Abs resulted in an even stronger inhibition of DGKα activity without affecting DGKα protein content (Fig. 1A). We then measured DGKα activity in anti-DGKα immunoprecipitates from Jurkat leukemia cells following costimulation with anti-CD3 and either anti-CD28 or anti-SLAM agonist Abs. Similar to the data on PBLs, DGKα enzymatic activity was strongly reduced upon 15 min costimulation via the TCR and either SLAM or CD28 and lasted for at least 1 h, without changes in DGKα protein content (Fig. 1B, 1C). Finally, to address the reported ambiguity of how anti-SLAM Abs may affect SLAM signaling, we used an alternative approach to induce SLAM signaling. We used a chimeric receptor featuring SLAM intracellular domain and the extracellular and transmembrane regions of the human IL-2 receptor α-chain coexpressed with SAP in BI-141 lymphocytes (30). Crosslinking of the chimeric receptor with TAC triggers SLAM signaling (30) and it was sufficient to induce a strong decrease of DGKα activity without changes in DGKα protein content (Fig. 1D). This result indicates that signals originating from the intracellular domain of SLAM lead to DGKα inhibition. Taken together, these observations indicate that upon costimulation of the TCR with either CD28 or SLAM, the enzymatic activity of DGKα undergoes a negative regulation, which likely contributes to the accumulation of DAG required for RasGRP–mediated activation of Ras and full T cell activation.

To verify whether this regulation was specific to DGKα, we first examined whether anti-CD3 costimulation with either anti-CD28 or anti-SLAM Abs regulated DGK′, which, along with DGKα, is highly expressed in T cells. We observed that neither CD3/CD28 nor CD3/SLAM costimulation of T cells did affect the enzymatic activity of DGK′ in anti-DGKα immunoprecipitates from either...
control or costimulated cells (Supplemental Fig. 1). These observations indicate that TCR activation specifically regulates DGKα enzymatic activity while not affecting DGKζ. To verify the contribution of DGKα regulation to the total cellular DGK activity, we measured DGK activity in whole-lymphocyte homogenates using exogenous substrates. Following 15 min TCR/CD28 costimulation of either PBLs or Jurkat cells, total DGK activity was not significantly affected, even when the costimulation was sufficient to activate ERK1/2 (Supplemental Fig. 2A, 2B). Conversely, upon 15 min TCR/SLAM costimulation, total DGK activity was significantly reduced (Supplemental Fig. 2C, 2D). Given the specific subcellular localization of DGK isoforms, these observations suggest that DGKα inhibition does not affect the bulk of DAG metabolism while selectively promoting DAG accumulation at specific compartments.

As DGKα recruitment from the cytoplasm to the plasma membrane is highly regulated both upon growth factor stimulation of epithelial cells and TCR/CD28-mediated costimulation of lymphocytes (20, 24), we assessed DGKα localization following costimulation of the TCR with either CD28 or SLAM. Both endogenous DGKα in CD3+ PBLs and YFP-DGKα transiently expressed in Jurkat cells localize diffusely in the nucleus and in the cytoplasm of unstimulated or TCR-stimulated cells. Upon 1 h costimulation of the TCR with either CD28 or SLAM, DGKα was almost entirely excluded from the nucleus and recruited to the cell periphery in both PBLs and Jurkat cells (Fig. 2A, 2B, Supplemental Fig. 3A, 3B). Whereas inhibition of DGKα enzymatic activity was an early event, starting 5 min following costimulation, reaching maximal inhibition at 15 min, and lasting up to 1 h (Fig. 1C), translocation of DGKα became detectable 15 min after costimulation, reached its maximum at 30 min, and lasted for several hours (Fig. 2B). To distinguish between plasma membrane and cytoplasmic localization, we labeled plasma membrane with either K-Ras-V12/A28 (31) or wheat germ agglutinin. Upon T cell costimulation, DGKα only partially colocalized with K-Ras-V12/A28 (Fig. 2A) or with wheat germ agglutinin (Supplemental Fig. 3B). Accordingly, ~10% of cytoplasmic DGKα sedimented in the 100,000 RCF fraction of CD3/CD28-costimulated Jurkat cells (Fig. 2C). These findings indicate that, upon lymphocyte activation, DGKα undergoes both negative regulation of its enzymatic activity and translocation from the nucleus to the cell periphery, although with different kinetics.

**Regulation of DGKα inhibition and recruitment to the cell periphery**

We explored whether translocation to the cell periphery and negative regulation of DGKα were regulated by common signaling pathways. DGKα activity and localization are regulated by Src-mediated tyrosine phosphorylation (21, 24, 25), calcium binding (17, 32), and D-3 phosphoinositides (33). Pharmacological inhibition of PLC by U73122 and calcium chelation by BAPTA-AM blunted DGKα translocation from the nucleus to the cell periphery induced by costimulation of TCR with either SLAM or CD28.
is restricted to SLAM-induced regulation of DGK activity and its translocation to the cell periphery. Conversely, inhibition of SFKs impaired only CD3/SLAM-induced translocation, indicating that phosphoinositide 3-kinases are not involved (Fig. 3A). Similarly, pharmacological inhibition of PLC and calcium signaling prevented the negative regulation of DGKα activity induced by TCR/SLAM costimulation (Fig. 3B). These data indicate that PLC activity and calcium release mediate both inhibition of DGKα activity and its translocation to the cell periphery. Conversely, inhibition of SFKs impaired specifically negative regulation of DGKα activity and its translocation to the cell periphery induced by TCR/SLAM costimulation, but not by TCR/CD28 costimulation (Fig. 3A, 3C), indicating that the requirement of SFKs is restricted to SLAM-induced regulation of DGKα. Despite that SAP overexpression regulates cdc42, IPA-3–mediated inhibition of PAK, a cdc42 effector, does not affect DGKα activity (Fig. 3A). Interestingly, PP2-mediated inhibition of Src family tyrosine kinases (SFKs) inhibited only CD3/SLAM-induced translocation. Conversely, wortmannin did not affect DGKα localization, indicating that phosphoinositide 3-kinases are not involved (Fig. 3A). Similarly, pharmacological inhibition of PLC and calcium signaling prevented the negative regulation of DGKα activity induced by TCR/SLAM costimulation (Fig. 3B). These data indicate that PLC activity and calcium release mediate both inhibition of DGKα activity and its translocation to the cell periphery. Conversely, inhibition of SFKs impaired specifically negative regulation of DGKα activity and its translocation to the cell periphery induced by TCR/SLAM costimulation, but not by TCR/CD28 costimulation (Fig. 3A, 3C), indicating that the requirement of SFKs is restricted to SLAM-induced regulation of DGKα. Despite that SAP overexpression regulates cdc42, IPA-3–mediated inhibition of PAK, a cdc42 effector, does not affect DGKα activity (Fig. 3B).

Upon SLAM engagement, SAP mediates the recruitment of Fyn, thereby promoting tyrosine phosphorylation of SLAM and activation of its downstream signaling (34). Thus, we investigated the role of SAP in negative regulation and membrane recruitment of DGKα. SAP expression was downregulated in Jurkat cells by lentiviral-mediated stable expression of a SAP-specific shRNA (Fig. 4A, 4B). In SAP-deficient Jurkat cells, but not in control shRNA cells, DGKα activity was not inhibited following stimulation of TCR and SLAM (Fig. 4A), consistent with the essential role of SAP in SLAM-induced signaling. Surprisingly, in SAP-deficient cells, DGKα activity was not inhibited by TCR/CD28 costimulation. This observation suggests that SAP is not only required for SLAM signaling, but may also play a more direct role in promoting negative regulation of DGKα enzymatic activity (Fig. 4B). Indeed, overexpression of SAP and myc-DGKα in Jurkat cells resulted in the reduction of DGKα activity by 60% as measured in anti-myc immunoprecipitates, whereas myc-DGKα protein content was not affected (Fig. 4C). Conversely, in the same assay SAP mutants unable to bind either SH3 domains (SAP-R78A) or both tyrosine-phosphorylated proteins and SH3 domains (SAP-R55L) (3, 35) failed to inhibit DGKα (Fig. 4C). These findings indicate that SAP overexpression is sufficient to inhibit DGKα through a mechanism that requires SH3-binding ability of SAP.

The sequence surrounding tyrosine 335 of DGKα (SIY335PSV) features a high similarity to the SAP-SH3 binding motif on SLAM (ITY281AQV) (36), suggesting that DGKα might bind directly to SAP. However, we could not detect a direct physical association between SAP and DGKα in a mammalian two-hybrid assay (Supplemental Fig. 4A) or in coimmunoprecipitation assays using transfected 293T cells (Supplemental Fig. 4B), even when the two proteins were coexpressed with SLAM and Fyn. Taken together, these results indicate that SAP does not inhibit DGKα by directly binding to it, but through the SAP-mediated recruitment of a yet unidentified SH3-containing protein.

The role of SAP in DGKα membrane recruitment in Jurkat cells was investigated by shRNA-mediated stable knockdown of SAP. SAP silencing selectively impaired the recruitment of DGKα to the cell periphery induced by TCR/SLAM costimulation, but not by TCR/CD28 costimulation (Fig. 4D). Similar results were obtained upon transient siRNA-mediated downregulation of SAP in Jurkat cells (Supplemental Fig. 4C). These observations suggest that SAP does not inhibit DGKα activity by directly binding to it, but through the SAP-mediated recruitment of a yet unidentified SH3-containing protein.

FIGURE 2. YFP-DGKα localization upon T cell stimulation. A, Jurkat A3 cells were transfected with YFP-DGKα (green) and after 24 h were serum starved for 2 h and seeded for 1 h on either poly-L-lysine, anti-CD3, anti-CD3 plus anti-SLAM, or anti-CD3 plus anti-CD28 (10 μg/ml each)-coated glass-bottom dishes and microscope images were acquired. Representative images are shown along with a quantification from three independent experiments. "p < 0.0005, t test versus control. Scale bar, 5 μm. B, Jurkat A3 cells were transfected with YFP-DGKα (green) and DS-Red-K Ras V12/A38 (red) and after 72 h were serum starved for 2 h and seeded on anti-CD3 plus anti-SLAM agonistic Ab (10 μg/ml each)-coated glass-bottom dishes and images were acquired at the indicated times. Representative images are shown. Scale bar, 5 μm. C, Jurkat A3 cells were stimulated with anti-CD3 and anti-CD28 agonistic Abs (10 μg/ml each) and homogenized 1 h later. The postnuclear and postmitochondrial fraction was separated by centrifugation (100,000 RCF) in a soluble fraction and in a membrane-associated fraction. One fiftieth of the soluble fraction and the entire membrane-associated fraction were analyzed by Western blotting for DGKα and LAT content.

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DGKα might be defective in the negative regulation of SAP-deficient Jurkat cells, T cells of XLP patients lacking SLAM or TCR/CD28. We therefore reasoned that, similarly to SLAM, SAP is required for DGKα recruitment to the cell periphery. In contrast, whereas SAP is required for TCR/CD28-induced enzymatic inhibition of DGKα, it is not essential for translocation of DGKα to the cell periphery. These findings indicate that the localization and enzymatic activity of DGKα are regulated through distinct processes and kinetics, although the mechanisms involved are still partially unknown.

**Inhibition of DGKα rescues the functional defects caused by SAP deficiency in XLP**

Collectively, these data demonstrate that SAP is essential for regulation of DGKα activity upon T cell activation via the TCR/SLAM or TCR/CD28. We therefore reasoned that, similarly to SAP-deficient Jurkat cells, T cells of XLP patients lacking functional SAP might be defective in the negative regulation of DGKα, thereby contributing to the defective lymphocyte responses observed in both XLP patients and in SAP-null mice. To address this hypothesis, we first characterized the signaling capacity of SAP-deficient Jurkat cells following stimulation via the TCR and CD28. We then assessed whether pharmacological inhibition of DGKα by R59949, or its siRNA-mediated downregulation, might rescue those aberrant T cell responses.

DAG-dependent recruitment of PKCθ to the plasma membrane is defective in T cells from SAP-null mice, it is potentiated upon SAP overexpression (7, 37) and it is negatively regulated by constitutive activation of DGKα (38). Consistently, in SAP-deficient Jurkat cells, PKCθ recruitment to the immune synapse with super Ag-loaded APCs was impaired (Fig. 5A). Both pharmacological inhibition (Fig. 5A) and siRNA-mediated silencing of DGKα (Fig. 5B) nearly completely rescued the defective translocation of PKCθ to the immune synapse observed in SAP-deficient Jurkat cells, pointing to a rescue of DAG-mediated signaling.

Upon TCR/CD28 costimulation, both T cells from XLP patients and Jurkat cells made SAP-deficient by siRNA-mediated downregulation exhibit defective ERK1/2 activation (5, 39), suggesting that DAG-mediated Ras-GTP signaling is impaired. Indeed, upon TCR/CD28 costimulation, Jurkat SAP-shRNA cells showed both a decrease in ERK1/2 phosphorylation and a marked reduction of Ras-GTP loading, as measured by Ras-GTP pull-down with GST-RBD (Fig. 5C). Pharmacological inhibition of DGKα with R59949 fully restored both ERK1/2 phosphorylation and Ras-GTP loading (Fig. 5C). These findings confirm that SAP is required for Ras activation in human T cells and provide further support to the hypothesis that negative regulation of DGKα is a critical step in the activation of the Ras pathway downstream of TCR/CD28. Interestingly, R59949 raised basal levels of ERK1/2 phosphorylation without significantly affecting Ras-GTP loading, suggesting that under these conditions ERK1/2 phosphorylation may be enhanced through a Ras-independent mechanism, likely through DAG-dependent PKCθ activation.

Activation of PKCθ and Ras pathways upon TCR/CD28 costimulation triggers NF-AT transcriptional activity, which plays a central role in cytokine production (8, 41). Moreover, NF-AT is activated upon SAP overexpression in Jurkat cells (42). Consistently, SAP downregulation in Jurkat cells impaired TCR/CD28-induced stimulation of NF-AT activity, as measured by luciferase reporter system (Fig. 5D, 5E). In SAP-deficient cells, pharmacological inhibition of DGK with 1 μM R59949 fully restored TCR/CD28-induced activation of NF-AT without affecting basal NF-AT activity (Fig. 5D), whereas siRNA-mediated DGKα silencing resulted only in partial rescue (Fig. 5E). These data suggest either that DGKα along with other R59949-sensitive DGKs mediate NF-AT activation downstream from SAP or that the low quantity of DGKα remaining after RNA interference may still transduce the signaling.

Upon T cell stimulation, activation of Ras, PKCθ, and NF-AT signaling pathways leads to IL-2 production (41, 43, 44), which has been reported to be reduced in lymphocytes from XLP patients (5). Indeed, in Jurkat cells, shRNA-mediated SAP silencing reduced TCR/CD28-induced IL-2 secretion (Fig. 5F). Pharmacological inhibition of DGKα by R59949 enhanced TCR/CD28-induced IL-2 production in control cells and fully rescued the defective IL-2 secretion of SAP-deficient Jurkat cells. These findings suggest that SAP-mediated negative regulation of DGKα is a key event in the modulation of T cell activation.

**Discussion**

In this study, we demonstrate that within minutes following costimulation of the TCR with either CD28 or SLAM, the enzymatic activity of DGKα, as assayed in immunoprecipitates in the pres-
FIGURE 4. SAP negatively regulates DGKα activity. A, Jurkat control-shRNA or Jurkat SAP-shRNA cells were stimulated for 15 min with 10 μg/ml anti-CD3 and anti-SLAM Abs and lysed. Anti-DGKα immunoprecipitates were assayed for DGK enzymatic activity while an aliquot of whole-cell lysate was analyzed by Western blot with anti-DGKα Ab to ensure equal loading and with anti-SAP Ab to verify the downregulation of SAP expression. A representative experiment is shown together with a graph of the mean ± SE of three independent experiments shown as percentage of control. *p < 0.05, t test versus control. B, Jurkat control-shRNA or Jurkat SAP-shRNA cells were stimulated for 15 min with 10 μg/ml anti-CD3 and (Figure legend continues)
ence of saturating DAG substrate concentration, undergoes a strong negative regulation without protein downregulation. This finding is surprising, given accumulating evidence that synthesis of PA is increased upon T cell stimulation (15, 16, 45) and that DGK activity is increased in whole-cell lysates from in vivo activated T cells (19). However, increased PA synthesis through DAG phosphorylation may depend on both positive regulation of one or more DGK isoforms and increased availability of DAG, whose production by PLCγ is increased upon TCR/CD28 costimulation (46). A parallel increase of DAG and PA levels upon TCR stimulation has been indeed observed (45, 47). Several pieces of evidence suggest that most of the PA generated upon T cell activation derives from phospholipase D2-mediated phospholipid hydrolysis and from DGKζ-mediated phosphorylation of DAG, whereas deletion of DGKα does not significantly affect PA production upon T cell stimulation (15, 16, 45). Nevertheless, recent genetic and biochemical data indicating that DGKα is a negative regulator of DAG-mediated TCR signaling (15, 17) are highly consistent with our finding that enzymatic activity of DGKα is reduced upon TCR costimulation with either CD28 or SLAM. This regulation appears to be isoform-specific, as DGKζ activity is unaffected by TCR triggering (Supplemental Fig. 1). Interestingly, the previous finding that stimulation of the sole TCR is not sufficient to promote sustained DAG signaling (48) is consistent with our observation that TCR activation in PBLs is not sufficient to inhibit DGKα activity in the absence of costimulation. Moreover, costimulation of TCR/CD28, compared with TCR alone, strongly enhances production of DAG but not of PA (15), suggesting a slowdown in the rate of DAG conversion to PA that is consistent with a negative regulation of DGK activity.

The molecular mechanisms underlying the negative regulation of DGKα have not yet been elucidated. In this study, we report that the adaptor function of SAP is required for DGKα inhibition induced by TCR costimulation with either SLAM or CD28. SAP is essential for SLAM tyrosine phosphorylation by recruiting the Src-related kinase FynT (3, 34); however, a growing body of evidence indicates that SAP is also involved in T cell responses to antigenic stimulation (2). Indeed, SAP binds directly to ITAM sequences of CD3ζ subunit (29), whereas TCR activation promotes the recruitment of SAP and SLAM family receptors to the signalosome (28, 29, 49). Furthermore, genetic deletion of SAP in mice results in the impairment of TCR/CD28-induced DAG-mediated activation of PKCθ and of downstream signaling events (7). Moreover, TCR/CD28-induced ERK1/2 activation and IL-2 production, which are both dependent on DAG-mediated activation of RasGRP, are impaired in T cells from SAP-deficient XLP patients (5). Intriguingly, SAP is physically associated to PKCθ, and it has been demonstrated that SAP overexpression, which is sufficient to inhibit DGKα, promotes PKCθ recruitment to the immune synapse (37). Finally, we and others have shown that, in Jurkat cells, SAP silencing impairs TCR-induced Ras-GTP loading, ERK1/2 activation, PKCθ recruitment, NF-AT activation, and IL-2 production (5–7). Taken together, these observations suggest that, upon TCR/CD28 costimulation, SAP is required for optimal DAG signaling. The finding that SAP is required for inhibition of DGKα might provide a mechanistic link between SAP and the regulation of DAG signaling. Thus, we propose that, upon stimulation of T cells from either SAP-deficient XLP patients or SAP-null mice, DGKα may inappropriately retain a high enzymatic activity, thereby converting DAG to PA and decreasing DAG signaling.

If this hypothesis holds true, we would expect that inhibition or downregulation of DGKα would rescue, at least partially, the defective signaling of SAP-deficient T cells. Accordingly, we observed that the inhibition of DGKα enzymatic activity in SAP-deficient Jurkat cells rescued defective DAG-dependent PKCθ membrane recruitment, Ras-GTP loading, ERK1/2 and NF-AT activation, and IL-2 production. These findings indicate that the excess of DGKα activity contributes to the defective signaling of SAP-deficient cells and, along with the demonstration that SAP overexpression inhibits DGKα, provide further support to the hypothesis that SAP negatively regulates DGKα. According to these findings, the negative regulation of DGKα activity represents a key event controlling the early phase of T cell activation by contributing to fine tuning of DAG levels required for appropriate signaling.

In this study, we observed that costimulation of the TCR with either SLAM or CD28 induces DGKα exit from the nucleus and accumulation in the cytoplasm with only partial localization at the plasma membrane. This finding appears to contrast previous studies reporting GFP-DGKα localization at the plasma membrane of CD3/CD28 costimulated Jurkat cells; however, according to the same authors, DGKα membrane translocation is rapid and transient and can be visualized in conditions that inhibit its relocalization to the cytoplasm (17, 21). Moreover, DGKα plasma membrane localization was clearly induced by stronger stimuli, such as the activation of ectopically overexpressed muscarinic receptor (20, 21, 50, 51) or Ag challenge in vivo (19). Further support to the hypothesis that enzymatic activity of DGKα regulates DAG level at the plasma membrane of T cells derives both from our finding that uncoupling of DGKα inhibition from TCR stimulation impairs PKCθ recruitment to the immune synapses (Fig. 5A) and from the observation that pharmacological inhibition of DGKα allows accumulation of DAG at the plasma membrane of T cells, thereby triggering activation of Ras signaling (52).

Interestingly, stimulation with either SLAM or TCR alone did not induce DGKα translocation from the nucleus, indicating that the concerted signaling via both receptors is required. Moreover, the finding that SAP, which is essential for SLAM tyrosine phosphorylation and signaling, is required for translocation induced exclusively by TCR/SLAM, but not by TCR/CD28, suggests that SAP may not directly regulate DGKα subcellular localization. Additionally, the fact that upon TCR/CD28 costimulation, SAP is required for inhibition of DGKα activity, but not anti-CD28 Abs and lysed. Anti-DGKα immunoprecipitates were assayed for DGK enzymatic activity while an aliquot of whole-cell lysate was assayed by Western blot with anti-DGKα Ab to ensure equal loading and with anti-SAP Ab to verify the downregulation of SAP expression. A representative experiment is shown together with a graph of the mean ± SE of three independent experiments shown as percentage of control. *p < 0.05, t test versus control. C. Jurkat A3 cells were transiently cotransfected with myc-DGKα and the indicated GFP-SAP mutants. After 48 h, cells were lysed and anti-myc immunoprecipitates were assayed for DGK enzymatic activity while an aliquot of whole-cell lysate was assayed by Western blot with anti-myc and anti-SAP Abs to verify transfection efficiency. A representative experiment is shown along with a graph showing the mean ± SE of three independent experiments shown as percentage of control. *p < 0.05, t test versus control. D. Jurkat control-shRNA and Jurkat SAP-shRNA cells were transfected with YFP-DGKα (green) and DS-Red-K-Ras V12/A38 (red). After 24 h, cells were serum starved for 2 h, seeded for 1 h on poly-1-lysine, anti-CD3 plus anti-SLAM, or anti-CD3 plus antiCD8 agonistic Ab (10 μg/ml each)-coated glass-bottom dishes, and images were acquired. Representative images are shown together with a quantification from three independent experiments. *p < 0.05, t test versus control. Scale bar, 5 μm.
FIGURE 5. DGKα inhibition rescues defective TCR-induced DAG-dependent signaling and IL-2 production of SAP-deficient T lymphocytes. A, Jurkat control-shRNA and Jurkat SAP-shRNA cells (T) were pretreated with R59949 (10 μM 30 min) incubated with super Ag-loaded Raji cells (APCs) for 15 min, fixed, and stained for PKCθ. Representative images are shown. Scale bar, 5 μm. Cells displaying PKCθ at the immune synapse were counted. The histogram shows data from three independent experiments as mean ± SE (*p < 0.05, t test). B, Jurkat control-shRNA and Jurkat SAP-shRNA cells (T) were transfected with DGKα-specific siRNA or control siRNA. After 72 h, cells were lysed and analyzed by Western blot with anti-DGKα and anti-actin Abs (left panel). At the same time cells were incubated with super Ag-loaded Raji cells (APCs) for 15 min, fixed, and stained for PKCθ. Cells displaying PKCθ at the synapse were counted (right panel). The histogram shows data from three independent experiments as mean ± SE (*p < 0.05, t test). C, Control shRNA Jurkat or SAP shRNA Jurkat cells were stimulated with 1 μg/ml anti-CD3 and 0.1 μg/ml anti-CD28 Abs in the presence or in absence of 1 μM R59949. After 15 min, cells were lysed and Ras-GTP was separated by pull-down with Raf-RBD and quantified by Western blotting with anti pan-Ras Ab. Total Ras, phospho-ERK1/2, and SAP contents were revealed in whole-cell lysates by Western blotting. D, Jurkat control-shRNA and Jurkat SAP-shRNA cells were transfected with a Dual-Luciferase NF-AT reporter system. After 48 h, cells were stimulated with 1 μg/ml anti-CD3 and anti-CD28 Abs in the presence or absence of 1 mM R59949. After 16 h stimulation, cells were lysed and analyzed for NF-AT–driven luciferase activity. Graph shows the mean ± SE of quadruplicates of a representative experiment. *<p < 0.05, t test versus control. E, Jurkat control-shRNA or Jurkat SAP-shRNA cells were transfected with a siRNA targeting DGKα or a control siRNA and a Dual-Luciferase NF-AT reporter system. After 48 h, cells were stimulated with 1 μg/ml anti-CD3 and anti-CD28 Abs. After 16 h stimulation, cells were lysed and analyzed for NF-AT–driven luciferase activity. Graph shows the mean ± SE of quadruplicates of a representative experiment. *<p < 0.05, t test versus control. F, Jurkat control-shRNA or Jurkat SAP-shRNA cells were stimulated with 1 μg/ml anti-CD3 and 0.1 μg/ml anti-CD28 Abs. After 72 h, cells were lysed and the amount of IL-2 released in the medium was measured by ELISA. Graph shows the mean ± SE of four replicates of a representative experiment. *p < 0.05, t test versus control.
for its exit from the nucleus, indicates that enzymatic activity and localization of DGKα are regulated independently of each other, as suggested also by the different kinetics of the two processes. Importantly, these findings also indicate that DGKα exit from the nucleus is not required for the inhibition of its enzymatic activity. The massive exit from the nucleus may reflect a potential increase in the availability of DGKα outside the nucleus for control of DAG signaling both at the plasma membrane and at intracellular vesicles. Indeed, several reports indicate a role of DGKs in T cells intracellular trafficking (50, 53, 54). Conversely, DGKα exit from the nucleus may contribute to regulate nuclear pools of DAG and PA. Indeed, several DGK isoforms have been reported to localize in the nucleus where they contribute to regulate transcription and cell cycle progression (55).

Previous evidence indicates that SFK-induced phosphorylation of DGKα on tyrosine 335 mediates its activation and membrane localization upon growth factor stimulation of epithelial and large cell lymphoma cells (24, 56). Moreover, in T cells DGKα phosphorylation by LCK on tyrosine 335 mediates recruitment of DGKα to the plasma membrane (21). Surprisingly, in our study pharmacological inhibition of SFKs did not affect either TCR/CD28-induced inhibition of DGKα or its exit from the nucleus, suggesting that both events are independent from SFK-mediated tyrosine phosphorylation of DGKα. Conversely, PP2 completely blocks DGKα inhibition and exit from the nucleus induced by CD3/SLAM, as SLAM signaling is dependent on Fyn tyrosine kinase.

The mechanism by which SAP regulates DGKα still remains to be elucidated. Based on the high similarity between the sequences surrounding tyrosine 335 of DGKα and tyrosine 281 of SLAM, we investigated the hypothesis that SAP may regulate DGKα by associating with it in a complex. However, we could not detect any direct or indirect physical interaction between the two proteins, even in a reconstituted association assay in a mammalian two-hybrid system. Our data demonstrate that SAP ability to inhibit DGKα requires the interaction with a yet unidentified SH3 domain-containing protein. The finding that inhibition of DGKα is independent of activity by SFKs suggests that the SAP interactor required for DGKα inhibition is not Fyn. The previous observation that SAP overexpression activates Cdc42 signaling by interacting with SH3-containing βPIX and independently of Fyn suggests that DGKα may be regulated by Cdc42-dependent PKA activation. However, the PK-specific inhibitor IPA-3 did not affect the inhibition of DGKα following TCR/SLAM costimulation (Fig. 3B). Finally, upon TCR stimulation of Jurkat cells, SAP silencing results in defective tyrosine phosphorylation of several proteins, including LAT and SLP76 (39). As both LAT and SLP76 are regulated independently of each other, as suggested also by the different kinetics of the two processes. Importantly, these findings also indicate that DGKα exit from the nucleus is not required for the inhibition of its enzymatic activity. The massive exit from the nucleus may reflect a potential increase in the availability of DGKα outside the nucleus for control of DAG signaling both at the plasma membrane and at intracellular vesicles. Indeed, several reports indicate a role of DGKs in T cells intracellular trafficking (50, 53, 54). Conversely, DGKα exit from the nucleus may contribute to regulate nuclear pools of DAG and PA. Indeed, several DGK isoforms have been reported to localize in the nucleus where they contribute to regulate transcription and cell cycle progression (55).

In conclusion, our findings suggest that the coordinated, but independent, control of DGKα enzymatic activity and of its localization regulates both its access to DAG and its rate of conversion to PA. Upon T cell stimulation, such a coordinated and complementary mechanism of regulation might finely tune the intensity and the duration of DAG-mediated signaling. Indeed, SAP silencing, by uncoupling TCR/CD28 costimulation from DGKα inhibition, results in the impairment of TCR/CD28-induced DAG-mediated signaling, providing further evidence that the SAP-mediated negative regulation of DGKα is crucial for the ability of T cells to trigger DAG-mediated responses.

Similar to cAMP signaling, which is triggered by G protein-coupled receptors by reciprocal regulation of both adenylate cyclase and phosphodiesterase activities (59), the findings presented in this study suggest that TCR/CD28 controls DAG signaling both by means of PLCγ activation and DGKα inhibition. Similarly, genetic and biochemical studies in Caenorhabditis elegans moto-neurons and murine hepatocytes showed that DAG-mediated signaling is controlled by G protein-coupled receptor-dependent reciprocal regulation of both PLC and DGKα (60–62).

In summary, our findings demonstrate that SAP-mediated DGKα inhibition is an early event in TCR signaling, which might be required for efficient T cell activation. The impaired regulation of DGKα activity in SAP-deficient lymphocytes may contribute to their defective TCR-induced responses, suggesting that pharmacological inhibition of DGKα could be useful in the treatment of certain manifestations of XLP.

Acknowledgments
M.C. Zhong and A. Veillette (Montréal, QC, Canada) provided BI-141 cells expressing IL-2R/SLAM chimera and SAP. M. Topham provided anti-DGKα Abs. P. Schwartzberg (National Institutes of Health, Bethesda, MD) provided GFP-SAP constructs.

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental figure 1. DGKζ activity is not modulated upon T cell activation

Jurkat A3 cells were stimulated for 15 min with 10 μg/ml of the indicated antibodies and lysed. Anti-DGKζ immunoprecipitates were assayed for DGK enzymatic activity while an aliquot of whole cell lysate was analyzed by western blot with anti-DGKζ antibody to ensure equal loading. A representative experiment is shown (right panel) together with a graph showing the mean ± SE of three independent experiments shown as percentage of control (left panel).
Supplemental figure 2. Modulation of total DGK activity during T cell activation

Jurkat A3 cells (A) and PBLs (B) were stimulated with 10 μg/ml anti-CD3 and anti-CD28 antibodies and homogenized after 15 minutes. Homogenates were analyzed for in-vitro total DGK enzymatic activity, and by western blot with anti DGKα, P-MAPK and MAPK antibodies. Graphs show mean ± SE of three samples from a representative experiment. Jurkat A3 cells (C) and PBLs (D) were stimulated with 10 μg/ml anti-CD3 and anti-SLAM antibodies and homogenized after 15 minutes. Homogenates were analyzed for in-vitro total DGK enzymatic activity, and by western blot with anti DGKα antibodies. Graphs show mean ± SE of three samples from a representative experiment. * t-test p<0.05
Supplemental figure 3. DGKα localization after TCR and SLAM co-stimulation.

(A) PBL were seeded on poly-lysine, stimulated with the indicated antibodies at a concentration of 10 μg/ml for 1 hour, fixed and stained for DGKα (green) and CD3 (red). Representative pictures are shown together with a quantification from three independent experiments, * t-test vs control p<0.05. Scale bar = 8 μm.

(B) Jurkat A3 cells were transiently transfected with YFP-DGKα (green), and following 72 hours were serum starved for 2 hours seeded on anti-CD3+anti-SLAM agonistic antibodies (10 μg/ml each) coated glass-bottom dishes and acquired at the indicated times. Alexafluor633-WGA (blue) was added 5 minutes before imaging. Representative pictures are shown. Scale bar = 8 μm.
Supplemental figure 4. SAP does not directly bind to DGKα

(A) Interaction between DGKα and SAP in mammalian two (three) hybrid system. Interaction plasmids containing full-length DGKα (v1) or the N-terminal portion of DGKα with the SLAM-like motif but without the kinase domain (v2) were cotransfected with the complementing SAP plasmid. No direct interaction was detectable, and addition of FynT did not circumvent this deficit. In comparison, SAP recognized the intracellular part of SLAM, and this interaction was markedly enhanced by the presence of FynT.

(B) 293T cells were transfected with empty vectors or vectors encoding myc-DGKα, SAP, SAP and SLAM or Fyn in the indicated combinations and after 48 hours cells were lysed. Myc-DGKα was immunoprecipitated with anti myc antibodies and immunoprecipitates together with total cell lysates were analyzed for myc-DGKα and SAP content.