Supplemental Data

The WAVE2 Complex Regulates Actin Cytoskeletal Reorganization and CRAC-Mediated Calcium Entry during T Cell Activation

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Supplemental Results

WAVE2 Is Phosphorylated after TCR Crosslinking

Recent experimental evidence in other cell types has demonstrated that WAVE family proteins become biochemically modified in response to receptor stimulation [S1]. We therefore asked if WAVE2 is modified after TCR crosslinking. In resting T cells (Jurkat, $CD4^+$, and $CD8^+$), WAVE2 migrated as a doublet by SDS-PAGE (Figure S2A). However, a single major slower-migrating form of WAVE2 was observed within 5–15 min post-TCR ligation, which persisted up to 30 min (Figure S2A). In order to determine if the mobility shift was a result of phosphorylation, we immunoprecipitated WAVE2 from unstimulated or TCR-stimulated Jurkat T cells and left the eluates untreated or treated them with shrimp alkaline phosphatase. Consistent with the idea that the slower migrating form results from phosphorylation, phosphatase treatment regenerated the faster migrating form of WAVE2 (Figure S2B). In contrast to previous findings [S2], we were unable to detect any tyrosine phosphorylation of WAVE2 in response to TCR ligation using three different (4G10, pTyr100, and pTyr102) anti-pTyr monoclonal antibodies (data not shown). Thus, we conclude that, in T cells, WAVE2 phosphorylation occurs primarily on serine and threonine residues. In fact, while we found that the phosphorylation of WAVE2 in response to TCR triggering is dependent on the proximal tyrosine kinases Lck and ZAP-70 (J.C.N. and D.D.B., unpublished data), we could bypass these kinases and produce the slower migrating WAVE2 protein by directly stimulating with PMA (Figure S2A). Because WAVE2 phosphorylation could be induced with PMA treatment, it was suspected that Erk and/or PKCs could be involved. We therefore tested the ability of the MEK inhibitor PD98059 and conventional PKC inhibitor GÖ6976 to inhibit WAVE2 phosphorylation. Whereas treatment with either inhibitor partially reduced WAVE2 phosphorylation, treatment with both inhibitors completely abrogated TCR-stimulated phosphorylation of WAVE2 (Figure S2C). These data demonstrate that WAVE2 is phosphorylated downstream of Erk and PKC in T cells after T cell receptor stimulation.

Supplemental Experimental Procedures

Reagents and Antibodies

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich. The antisera against PLCγ1, LAT, and ZAP-70 have been described previously [S3]. Polyclonal rabbit antibodies were raised against GST fusion proteins containing amino acids 185–265 and 350–498 of WAVE2 and 100-250 of Abi-1, which recognizes both Abi-1 and Abi-2. Similarly, polyclonal rabbit antibodies against HEM-1 and PIR121 were generated using synthetic peptides corresponding to amino acids 518–555 of PIR121 and 1118–1127 of HEM-1. The Abi-2 antibody has been previously described [S4] and was a gift from Dr. Ann Marie Pendergast, Duke University. Antibodies against ERK1/2, pERK1/2 (T202/Y204), pJNK1/2 (T183/Y185), and pPLCγ1 (Y783) were from Cell Signaling Technology, and a total JNK (C-17) was obtained from Santa Cruz. The OKT3 mAb antibody was obtained from the Mayo Pharmacy, the anti-CD28 mAb was purchased from BD Biosciences, and the anti-phosphotyrosine mAb (4G10) and antibody against WASp were purchased from Upstate Biotechnology. Other anti-phosphotyrosine mAbs (pTyr100 and pTyr102) were purchased from Cell Signaling. The antibody for GAPDH was purchased from Chemicon International, Inc.

RT-PCR Analysis

RNA was harvested using the TRIzol method (Invitrogen), and cDNA was generated with the First-Strand cDNA synthesis kit from Amersham Biosciences (Piscataway, NJ). Oligonucleotides for detecting WAVE1 and WAVE3 transcripts have been described previously [S5]. The following oligonucleotides were used to analyze WAVE2 expression levels (WAVE2 5′: 5′-ATAATCCAAATCGAGG GAAT-3′ and WAVE2 3′: 5′-TTGTCCTCGGAGAAGGAAGG-3′). RT-PCR was performed using 1 μl of cDNA in a 50 μl PCR reaction using 25 cycles for amplification. DNA bands were resolved on 1.25% agarose gel and visualized with ethidium bromide staining.

Plasmids and Cloning

The parental (pFRT-H1P) and GFP expressing (pCMS3.EGFP.H1P) vectors used for shRNA silencing have been described previously [S3]. The following 19 nucleotide sequences were generated to target human WAVE2 (A: 5′-GAGAAGAGAAAGCACAGGA-3′; B: 5′-CAC CCGAAAAGCCTTCAGA-3′), WASp (5′-GAGTGGCTGAGTTACTTGC-3′), HEM-1 (A: 5′-TACCACCTTCAGTACTTGG-3′; B: 5′-TCTACAACA AGAACATTGA-3′). A cherry-shRNA suppression vector was created by replacing the GFP in the parental pCMS3.EGFP.H1P vector with monomeric cherry fluorescent protein [S6]. The expression constructs for WT and LI PLCγ1 have been previously described [S7].

Cell Culture and Transfection

Primary human $CD4^+$ T cells, Jurkat T cells, previously described Jurkat T cells stably expressing GFP-actin [S8], A20 B cells, NALM6 B cells, and Raji B cells were grown in RPMI-1640 supplemented with 5% fetal bovine serum, 5% fetal calf serum, and 4 mM L-glutamine. $CD4^+$ and $CD8^+$ T cell clones were cultured as previously described [S9]. DO11.10 T cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, 1 mM sodium pyruvate, and nonessential amino acids (Gibco). DO11.10 secondary T cells were prepared from spleen and lymph node cells that had been cultured at 2–3 $\times$ 10^6 cells/ml with 1 μg/ml ovalbumin peptide (OVA 323–339) for two days and then cultured at 1 $\times$ 10^6 with rIL-2 50 U/ml overnight. Transient transfections in Jurkat were performed using 1 $\times$ 10^7 cells per sample along with 30–40 μg of plasmid DNA as previously described [S10]. Transfected Jurkat cells were used after 18–24 hr for expression and 48–72 hr for suppression.

Luciferase Reporter Assays

Jurkat cells (1 $\times$ 10^7 cells) were transfected with the indicated suppression vectors and distributed in triplicate into 24-well plates and stimulated with soluble OKT3 and CD28 antibody at a 1 μg/ml concentration each as indicated. Reporter plasmids for IL-2 [S3], NFAT/AP-1 [S9], AP-1 [S10], NFκB [S11], and NFAT [S10], have all been previously described. All samples were harvested and prepared for luciferase assays according to the protocol suggested by the manufacturer (Promega), and luciferase activity was measured with a Model LB9507 Lumat luminometer (Berthold Systems). All reporter assays were co-transfected with a pRL-TK reporter plasmid to control for inter-sample variations in transfection efficiency. Firefly and pRL-TK-derived *Renilla* luciferase activities were measured in each sample with a Dual Luciferase Assay kit (Promega).

Cell Stimulation and Immunoblot Analysis

For the stimulation timecourse studies in Jurkat T cells, 2 $\times$ 10^6 untransfected or transfected Jurkat T cells were stained on ice with 5 μg/ml anti-CD3 (OKT3, mAb) and then crosslinked using goat anti-mouse over the indicated time course at 37ºC. After each time point the cells were immediately washed in ice cold PBS and lysed in NP-40 lysis buffer (20 mM HEPES [pH 7.9], 100 mM NaCl, 5 mM EDTA, 0.5 mM CaCl, 1% NP-40, 1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM Na_3VO_4) for 10 min on ice. Lysates

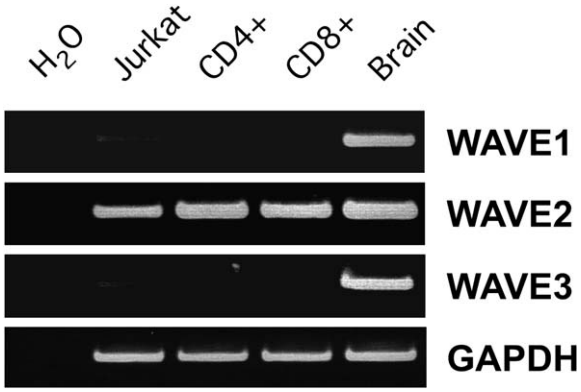

Figure S1. WAVE2 Is the Major Isoform Present in T Cells

cDNA was generated from RNA isolated from Jurkat T cells, primary human $CD4^+$ T cells, clonal $CD8^+$ T cells, and human brain. RT-PCR was performed to analyze the expression levels of WAVE1–WAVE3 and GAPDH in the various tissue samples.

were clarified by centrifugation at 18,000 × g for 5 min at 4ºC and then transferred to antibody-coated beads. The protein complexes were then washed twice with NP-40 lysis buffer, eluted in 60 μl of SDS-sample buffer, resolved by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). In some experiments, Jurkat T cells were preincubated for 30 min with either DMSO as a control, the MEK1 inhibitor PD98059 (10 μM) or GÖ6976 (2 μM), stimulated through the TCR, lysed, and WAVE2 was immunoprecipitated. Immunoprecipitates were then left untreated or treated with shrimp alkaline phosphatase (Boeringer Manheim, St. Louis, MO) for 15 min at room temperature in the absence or presence of vanadate. In cases in which whole-cell lysates were prepared, 100 μg of protein was resolved by SDS-PAGE. For immunoblots, mAbs were detected using goat anti-mouse IgG coupled to horseradish peroxidase (Santa Cruz), and polyclonal rabbit antisera were detected using Protein-A-linked to horseradish peroxidase (Amersham) and SuperSignal (Pierce, Rockford, IL). WAVE2 protein levels relative to ZAP-70 levels were quantified using an Ambis Radioanalytical Imaging System (San Diego, CA).

Immunofluorescence Microscopy

B cell-T cell conjugates were formed essentially as described previously [S12]. Briefly, NALM6, Raji, or A20 cells were stained with CellTracker Blue CMAC (7-amino-4-chloromethylcoumarin, Molecular Probes) and pulsed with or without 2 μg/ml SEE, 10 μg/ml OVA peptide (AA 323–339), or 2 μg/ml of a cocktail of Staphylococcal superantigens (SEA, SEB, SEC3, SEE; Toxin Technologies). B cells were centrifuged together with the same number of T cells, incubated at 37ºC for 15 min, plated onto poly-L-lysine-coated coverslips, and fixed with 4% paraformaldehyde/PBS for 15 min at room temperature. Fixed cells were quenched with 50 mM NH_4Cl and permeabilized in 0.3% Triton X-100. Blocking and antibody incubations were performed in PBS/0.05% saponin/0.25% fish skin gelatin. Slides were labeled with rabbit anti-WAVE2 followed by goat anti-rabbit FITC (Molecular Probes). F-actin was visualized with rhodamine phalloidin (Molecular Probes). Coverslips were mounted in Mowiol 4-88 (Hoeschst Celanese) containing 10% 1,4-diazobicyclo [2.2.2] octane. Quantification of F-actin polarization was performed by an individual blinded to the experimental conditions. To minimize bias, 50 conjugates were chosen at random, based upon DIC and CMAC images, disregarding cell morphology or protein distribution, and only conjugates consisting of 1 green (GFP-transfected) T cell and 1 blue (CMAC stained) B cell were scored. Those conjugates showing a distinct, bright band of labeling at the cell-cell contact site were scored as positive. Typically, this band was much brighter

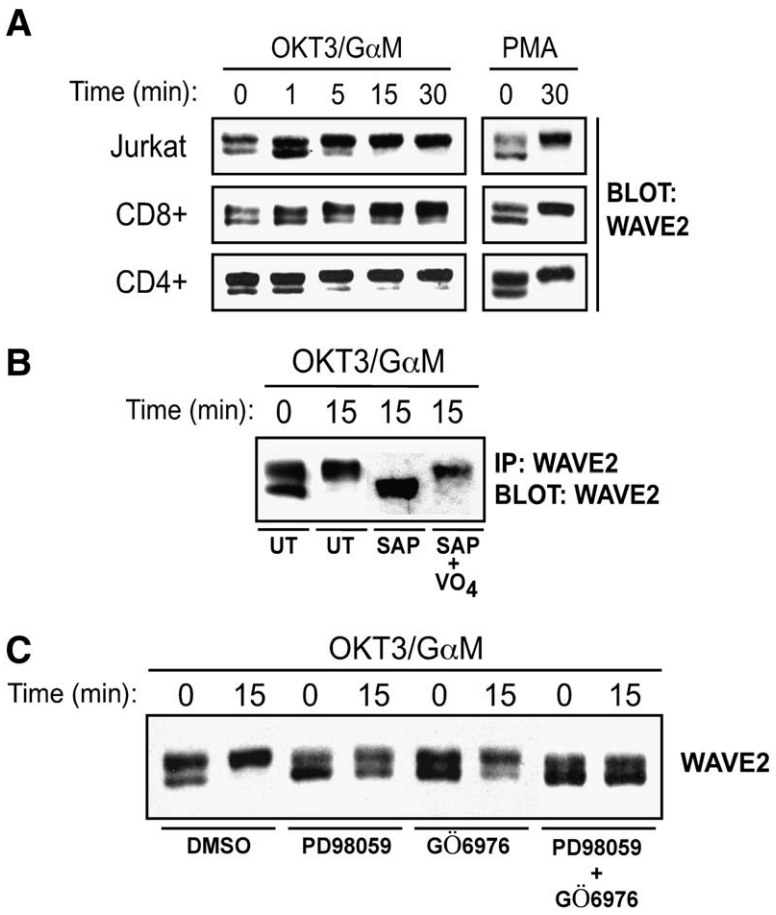

Figure S2. WAVE2 Is Phosphorylated in Response to TCR Ligation

(A) The indicated cell types were stimulated over time (above lane) or with PMA for 30 min. Cell lysates were prepared and separated by SDS-PAGE and probed for WAVE2.

(B) Jurkat T cells were stimulated as indicated (above lane) and WAVE2 was immunoprecipitated, then left untreated or treated with shrimp alkaline phosphatase for 15 min at room temperature in the presence or absence of vanadate (VO4) and WAVE2 protein was visualized by immunoblot.

(C) Jurkat T cells were treated with the indicated pharmacological inhibitors for 30 min (below lane) and stimulated through the TCR for 0 or 15 min. WAVE2 protein was then analyzed by immunoblot.

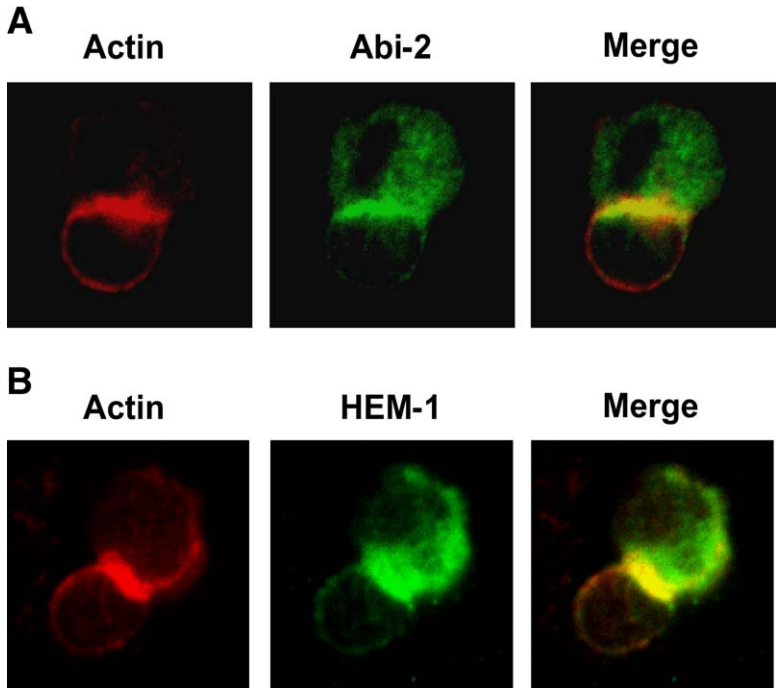

Figure S3. WAVE2 Complex Members Localize to the IS

(A) Primary human CD4$^+$ T cells were allowed to form conjugates with superantigen cocktail pulsed Raji B cells. Conjugated cells were bound to poly-L-lysine coated coverslips, fixed, and stained with antibody against Abi-2 (green) and rhodamine-phalloidin (red). (B) Same as in (A), except cells were stained with antibody against HEM-1 (green).

than any other portions of either cell, however, where necessary, the pixel intensity was determined, and only those interfaces with pixel intensity greater than the sum of the two cell surfaces away from the interface were scored as polarized.

Conjugate Analysis

Conjugate assays were performed essentially as described previously [S8]. Briefly, NALM6 cells were stained with hydroethidene according to manufacturers directions. After quenching with media

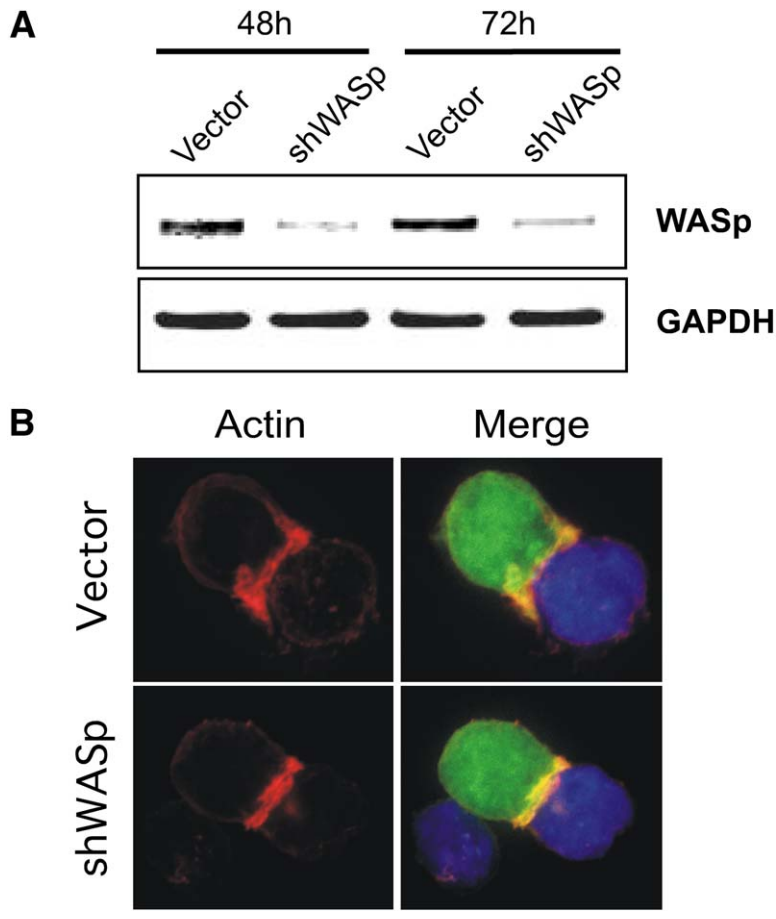

Figure S4. WASp Suppression Does Not Affect Actin Polymerization at the IS

(A) Jurkat T cells were transfected with shWASp suppression vector containing a separate GFP transcriptional cassette or a control vector. At the 48 and 72 hr time points, cells were lysed and proteins were separated by SDS-PAGE, transferred, and subsequently blotted for WASp and GAPDH. Transfection efficiencies of >85% led to a 70% reduction in total WASp protein levels. (B) Cells were transfected as in (A), and after 72 hr were incubated with SEE-pulsed NALM6 B cells (blue). Conjugates were bound to poly-L-lysine coated coverslips, fixed, and stained with rhodamine-phalloidan (red) to visualize accumulation of F-actin.

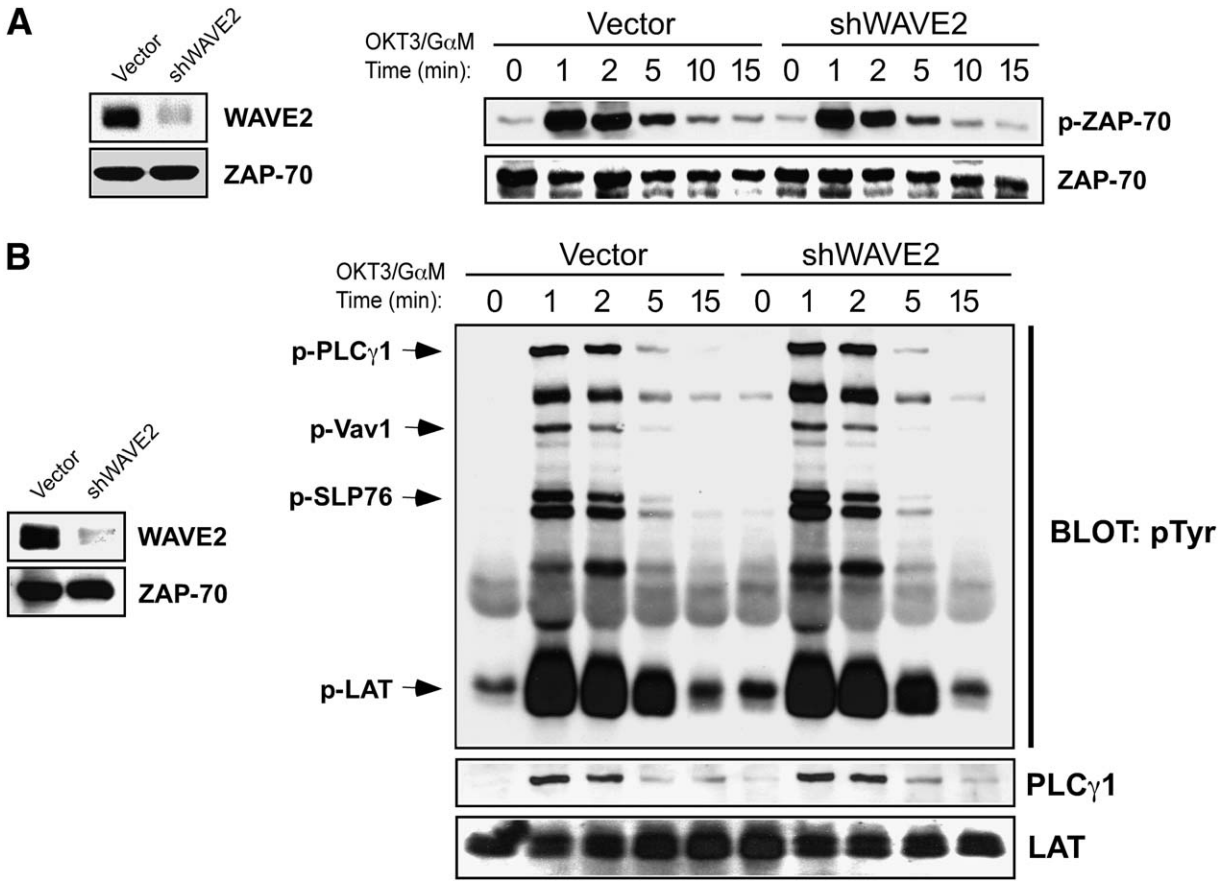

Figure S5. WAVE2 Suppression Does Not Affect Proximal Tyrosine Kinases or Formation of the LAT Signalosome

(A) Jurkat T cells were transfected with vector control or shWAVE2 suppression vector. After 72 hr, cells were stimulated with OKT3 and goat anti-mouse antibody for the indicated time points. IPs against ZAP-70 were prepared and bound proteins were separated by SDS-PAGE and transferred. Tyrosine phosphorylation of ZAP-70 was determined by blotting for pTyr and total ZAP-70.

(B) Cells were transfected and stimulated as in (A). IPs against LAT were prepared and bound proteins were separated by SDS-PAGE and transferred. Tyrosine phosphorylated proteins were analyzed by blotting for pTyr. Association of PLCγ1 with LAT was then determined by immunoblot.

containing serum, cells were incubated in the presence or absence of 2 μg/ml SEE for 1 hr, washed, and resuspended at 1×10^6 cells/ml in RPMI. Jurkat T cells expressing GFP alone or together with WAVE2 shRNA were also resuspended at 1×10^6 cells/ml in RPMI. For conjugation, equal volumes of B and T cells were pelleted together and then incubated at 37ºC for 10–15 min. Cells were vortexed for 5–10 s and then fixed by adding an equal volume of 4% paraformaldehyde. The relative proportion of red, green, and red/green events in each tube was determined by two-color flow cytometric analysis by using a FACScalibur flow cytometer (BD Biosciences). The number of gated events counted per sample was at least 15,000.

Adhesion Assays

Adhesion assays were performed as previously described [S13, S14]. Briefly, Jurkat T cells were transfected with the indicated GFP-suppression vector, and adhesion analysis was performed 48–72 hr later with a 96-well plate precoated with 0.3 μg/well fibronectin. For TCR stimulation, the cells were preincubated with OKT3 and then added to wells containing secondary anti-IgG as a crosslinker. Adhesion was quantified by flow cytometry as previously described [S13, S14].

Live Cell Imaging

Jurkat T cells were transfected with cherry-shRNA suppression vectors and were used for imaging 48 hr post-transfection. Time-lapse images of actin dynamics were collected on a Zeiss LSM-510 laser scanning confocal microscope using a 63× water objective. Chambered 4-well Lab-Tek borosilicate coverglasses (Nalge Nunc Inc., Naperville IL) were treated with 20 μg/ml OKT3 (anti-CD3) in PBS overnight at 4ºC with rocking, blocked with PBS containing 1% BSA for 3 hr, and then rinsed four times with PBS. Each slide was preheated on a 37ºC heated stage and spreading assays were initiated by adding 5 μl of a concentrated cell suspension (2×10^6 cells/ml in serum-free RPMI medium) to the slide well containing PBS. Images just above the plane of the coverglass were collected every 5 s and were sequenced into videos using QuickTime v.7.0.1 (Apple Computer, Inc.).

Calcium Mobilization

Jurkat T cells were transfected with the indicated control or shRNA suppression vectors for 72 hr, loaded with Indo-1 acetoxy-methyl ester (Molecular Probes), and stimulated with 50 ng/ml OKT3 mAb cross-linked with goat anti-mouse. Changes in intracellular [Ca^{2+}] were determined by flow cytometry as described previously [S15].

Single Cell Calcium Analysis

Jurkat T cells were loaded with the cell permeant calcium indicator fura-2 AM (3.0 μM, Molecular Probes, USA) in RPMI medium for 15 min at room temperature (25ºC). For the final 10 min of Fura-2 loading, OKT3 (5 μg/ml) was added to the incubation mixture. Cell suspensions containing Fura-2 and OKT3 were placed into the recording chamber on an inverted fluorescence microscope (Nikon) and

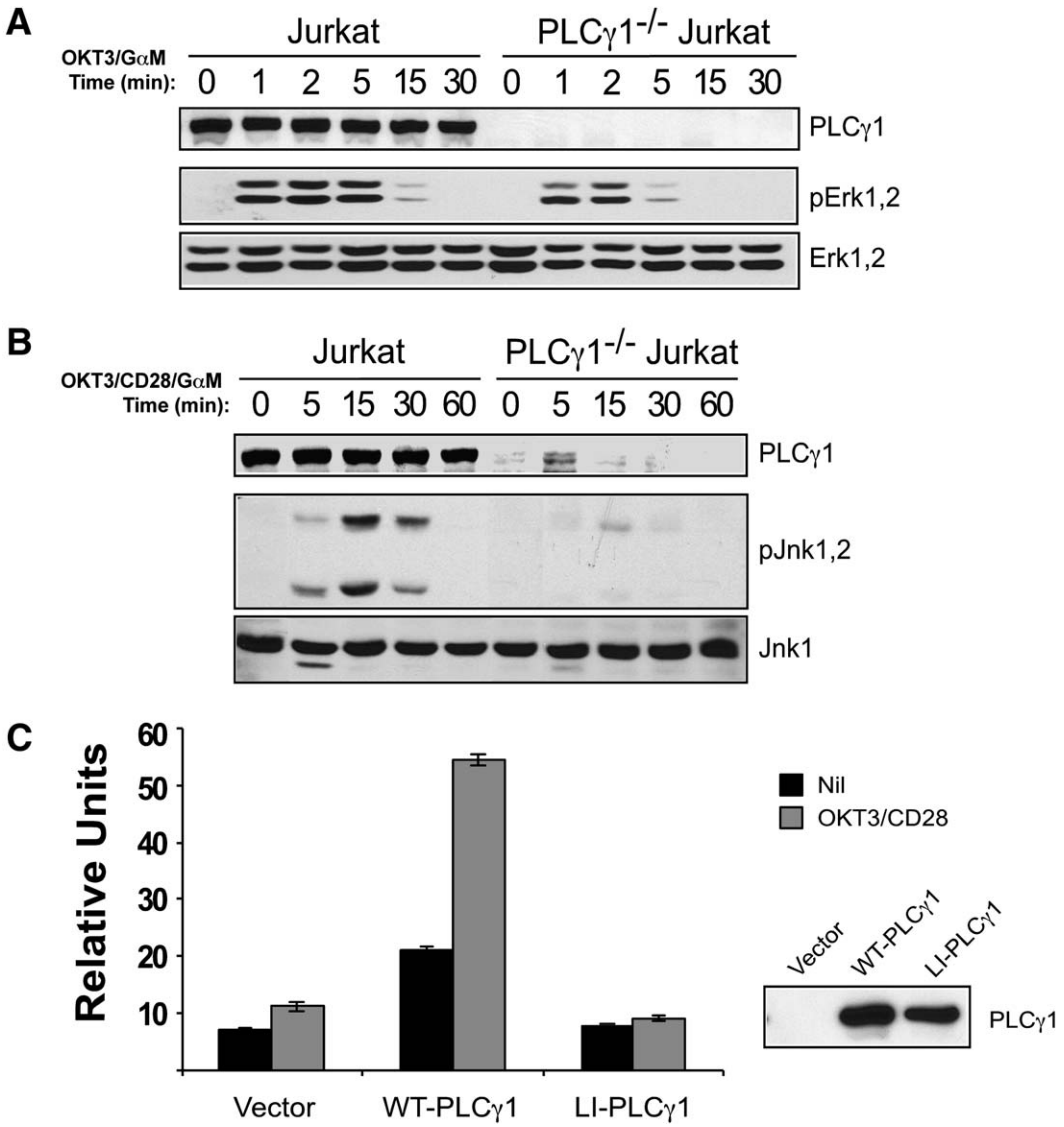

Figure S6. Functional PLCγ1 Is Required for the Activation of Erk and Jnk

(A) Jurkat T cells or PLCγ1$^{-/-}$ Jurkat T cells were stimulated with OKT3 and goat anti-mouse antibody for the indicated time points. Whole-cell extracts were prepared and proteins were separated by SDS-PAGE, transferred, and blotted for the phosphorylation of Erk1 and Erk2.

(B) Same as in (A), except cells were stimulated with OKT3 and anti-CD28 and blotted for the phosphorylation of Jnk1 and Jnk2.

(C) PLCγ1$^{-/-}$ Jurkat T cells were transfected with an AP-1 reporter construct and with either vector control or expression constructs for WT PLCγ1 or lipase inactive (LI) PLCγ1. After 24 hr, cells were either left unstimulated (Nil) or stimulated with OKT3 and anti-CD28 (OKT3/CD28) for 5 hr. Luciferase activity was measured and normalized to TK-Renilla readings.

allowed to adhere to Poly-L-lysine (100 μg/ml, Sigma) treated coverslips for 5 min in a solution which contained 155 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Hepes (pH 7.4). Excess fura-2 AM (Molecular Probes) and OKT3 were removed by perfusing the chamber with additional extracellular solution. Before stimulation, the chamber was perfused with Ca^{2+}-free bath solution containing 155 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$ and 0.5 mM EGTA, 10 mM glucose and 10 mM Hepes (pH 7.4). Intracellular Ca^{2+} mobilization was initiated by addition of goat anti-mouse IgG in Ca^{2+}-free bath solution or the addition of C305 monoclonal antibody. The initial Ca^{2+} transient observed in Ca^{2+}-free solution is due to Ca^{2+} release from stores. The activation state of calcium entry channels was then examined by replacing the calcium-free solution with normal (Ca^{2+} containing) bath solution. Fura-2 fluorescence of individual cells was measured by digital imaging microscopy as previously described [S16] and plotted as the 340 nM/380 nM ratio. Where indicated, thapsigargin was used to block SERCA pump activity and calcium mobilization was analyzed as described above.

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