Recruitment of Sprouty1 to Immune Synapse Regulates T Cell Receptor Signaling[1]

Jun Sung Lee,[2]* Ji Eun Lee,[2]* Yu Mi Oh,* Jong Bae Park,* Heonsik Choi,‡ Chung Yeon Choi,† In-Hoo Kim,† Seung Hoon Lee,* and Kyungho Choi[3]*

TCR stimulation not only initiates positive signals for T cell activation, but also induces negative signals that down-regulate T cells. We previously reported that Sprouty1, a negative regulator of Ras-MAPK pathway of receptor tyrosine kinases, was induced by TCR signal and inhibited TCR signaling in CD4$^+$ T cell clones. In this study, we addressed the mechanism underlying Sprouty1 inhibition of T cells. When overexpressed in Jurkat T cells, Sprouty1 inhibited TCR signal-induced IL-2 transcription, and also AP-1, NFAT, and NF-κB activation, which suggests that Sprouty1 acts at proximal TCR signalosome. Accordingly, we found that Sprouty1 translocated to immune synapse upon TCR engagement in both Jurkat cells and activated primary T cells and interacted with various signaling molecules in the TCR signalosome, such as linker for activation of T cells (LAT), phospholipase C-γ1 (PLC-γ1), c-Cbl/Cbl-b, and HPK1. Sprouty1 inhibited LAT phosphorylation, leading to decreased MAPK activation and IL-2 production. Deletion of C-terminal 54 amino acids in Sprouty1 abolished its inhibitory effect and this deletion mutant was unable to translocate to immune synapse and interact with LAT. Overall, our data suggest that Sprouty1 induced by TCR signal negatively regulates further TCR signaling by interacting with proximal signaling molecules in immune synapse, providing a novel regulatory mechanism of T cells. *The Journal of Immunology,* **2009, 183: 7178–7186.**

After T cells are activated by Ag engagement, their response should be turned off to maintain homeostasis of T cell population in vivo (1). Molecularly, this kind of negative regulation is driven partly by T cell activation itself, i.e., TCR signal transduction. For example, TCR signaling induces expression of FasL and TNF-α receptor on the surface of T cells, making them susceptible to apoptotic cell death (2). CTLA-4 and programmed cell death 1 (PD-1),[4] coinhibitory receptors, are induced by TCR signaling and inhibit further proliferation of T cells (3, 4). TCR signaling also induces synthesis and secretion of inhibitory cytokines, such as IL-10 and TGF-β, in some subsets of T cells (5, 6). These inhibitory feedback machineries have been clearly documented in vivo by lymphoproliferative disorders shown in each knockout mouse (7–12). Even though these inhibitory machineries from the outside of T cells are well documented, not many intracellular inhibitory molecules up-regulated by TCR signaling have been reported.

Previously, we reported that Sprouty1, an intracellular signaling adaptor, was induced by TCR signaling and that overexpressed Sprouty1 inhibited TCR signaling in fully differentiated murine CD4$^+$ T cell clones (13). Sproutys have been known to be negative regulators of many receptor tyrosine kinases, such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). Sprouty was first discovered in Drosophila as an inhibitor of FGF receptor signaling (14). Sprouty-null mutants had increased lung budding due to excessive FGF signal, from which the name Sprouty was derived. Subsequently, its inhibitory effect was extended onto epidermal growth factor (EGF), Torso, and Sevenless (15, 16). Four homologues (Sprouty1–4) have been found in mammalian cells. Their inhibitory effect was mainly ascribed to their ability to inhibit Ras-MAPK pathway at various levels (17–19). Importantly, Sprouty2 and -4 are induced by growth factor signals such as FGF and EGF, indicating that some Sprouty-homologues are negative feedback regulators of receptor tyrosine kinase signaling (20). In T cells, induction kinetics of Sprouty1 was delayed, showing its profound accumulation after 16 h of initial TCR signal, a time frame comparable to that of CTLA4 or PD-1. This led to an assumption that Sprouty1 may act as a negative feedback inhibitor of TCR signaling to prevent further stimulation of T cells.

The molecular mechanism of inhibitory effects of Sproutys has been studied mainly in FGF receptor (FGFR) signaling. Although there is general consensus that Sproutys specifically inhibit Ras-MAPK pathway, whether the action site of Sproutys is located before Ras (21), at Ras (17), or after Ras level (22) is not clear. The most attention-drawing scenario was that FGF stimulation induces phosphorylation of tyrosine 53 in Sprouty1 and tyrosine 55 in Sprouty2, and the phosphorylated Sprouty1/2 binds to Grb2-son of Sevenless (SOS) complex to sequester it from FRS2, preventing further signal transduction (21). However, this mechanism remains controversial because it was shown that Sprouty2 constitutively

*Specific Organs Cancer Branch and †Molecular Imaging and Therapy Branch, Research Institute National Cancer Center, Gyeonggi-do, Korea; and ‡Division of Molecular Immunology, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037

Received for publication November 13, 2008. Accepted for publication September 26, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from National Cancer Center (NCC-0710060) and the Innovative Research Institute for Cell Therapy (A062260), Republic of Korea.

[2] These authors equally contributed to this work.

[3] Address correspondence and reprint requests to Dr. Kyungho Choi, Research Institute National Cancer Center, 809 Madu1-dong, Ilsandong-gu, Goyang-si, Gyeonggi-do, 410-769, Republic of Korea. E-mail address: kchoi@ncc.re.kr

[4] Abbreviations used in this paper: PD-1, programmed cell death 1; Cbl-b, casitas B-lineage lymphoma b; c-Cbl, casitas B-lineage lymphoma; EGF, epidermal growth factor; EGFR, EGF receptor; FGF, fibroblast growth factor; FGFR, FGF receptor; HA, hemagglutinin; HPK1, hematopoietic progenitor kinase 1; IP_3, inositol 3,4,5-triphosphate; LAT, linker for activation of T cells; PLC-γ1, phospholipase C-γ1; SEE, staphylococcal enterotoxin E; SOS, son of Sevenless; VEGF, vascular endothelial growth factor.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803799

interacts with Grb2 independent of tyrosine 55 phosphorylation and that phosphorylation-defective mutants of Sprouty2, which are unable to inhibit FGFR signaling, still bind to Grb2 (17, 23). In other studies of VEGF receptor signaling, Sprouty4 was shown to directly bind to Raf1 and block its phosphorylation independent of Ras (24).

TCR signal transduction cascade begins with phosphorylation of ζ-chain of CD3 complex coupled to TCR α- and β-chains. Subsequently, the tyrosine kinase, ZAP70, binds to phosphorylated ζ-chain and becomes activated. Activated ZAP70 phosphorylates an adaptor called LAT, which recruits Grb2, Grb2-related adaptor protein 2 (GADS), Src homology 2 domain containing leukocyte protein of 76kDa (SLP76), and phospholipase C-γ1 (PLC-γ1). PLC-γ1 in turn hydrolyzes PIP_2 into inositol 3,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 activates calcium-NFAT pathway, while diacylglycerol activates PKCθ-NFκB and Ras-GRP-Ras-MAPK pathways (25).

Since our initial discovery of the inhibitory role of Sprouty1 in T cell clones, its molecular action mechanism has been largely unknown in T cells. In this study, using Jurkat T cells and activated primary T cells, we demonstrate that Sprouty1, recruited to TCR signalosome by TCR signal, interacts with various signaling molecules, such as LAT, HPK1, PLC-γ1, and c-Cbl/Cbl-b. Overexpressed Sprouty1 inhibited TCR-induced LAT phosphorylation, leading to decreased Erk activation and IL-2 transcription. This inhibitory activity of Sprouty1 was abolished following the deletion of its C-terminal 54 amino acids. This deletion also abolished the localization of Sprouty1 to immune synapse and the interaction between Sprouty1 and LAT. These results suggest that Sprouty1 induced by TCR signaling may inhibit further TCR signals by interacting with proximal signaling molecules including LAT, providing a novel molecular mechanism of Sprouty1 action in T cells.

Materials and Methods

Mice and cell preparation

C57BL6 mice were purchased from Japan SLC. Pigeon cytochrome C-specific TCR (5C.C7)-transgenic, $Rag2^{-/-}$ mice were obtained from National Institute of Allergy and Infectious Diseases/Taconic Repository. The mice were bred in specific pathogen-free animal facility at Research Institute National Cancer Center, Korea and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee. Primary murine $CD4^+$ T cells were purified from lymph nodes and spleens of C57BL6 mice by positive selection using anti-CD4-microbeads (Miltenyi Biotec). For activation of primary T cells, the cells were incubated with plate-bound anti-mouse CD3ε (145-2C11; 1 μg/ml; BD Biosciences) in the presence of soluble anti-mouse CD28 (37.51; 1/2500 dilution of ascites) for 24 to 48 h. For TAT-protein transduction, cells from lymph nodes of 5C.C7 TCR-transgenic mice were used as naive $CD4^+$ T cells without further purification (usually more than 90% of purity).

Cell lines

Jurkat T cell Clone E6-1 (American Type Culture Collection (ATCC)) was maintained in RPMI 1640 (HyClone) supplemented with 10% FBS, penicillin (1000U/ml), and streptomycin (1000U/ml). Tet-off Jurkat T cells (Clontech) were cultured in RPMI 1640 (HyClone) supplemented with 10% Tet-system-approved FBS, 4 mM glutamine, penicillin (1000U/ml), and streptomycin (1000U/ml). Raji cells (ATCC) were maintained in DMEM (HyClone) with 10% FBS, penicillin (1000U/ml), and streptomycin (1000U/ml).

Antibodies

Abs for T cell stimulation (anti-CD3ε and anti-CD28) were from BD Biosciences. The sources of Abs used for Western blots and immunoprecipitations were as follows: mouse monoclonal anti-phosphotyrosine (4G10), anti-LAT, anti-phospho-LAT (Y171), anti-ZAP70, and anti-ERK (Upstate Biotechnology); anti-PLC-γ1, anti-phospho-ZAP70 (Y319), anti-HPK1, anti-c-Cbl, and anti-Cbl-b (Santa Cruz Biotechnology); anti-phospho-LAT (Y171, Y191) and anti-phospho-PLC-γ1 (Y783) (Cell Signaling Technology); anti-hemagglutinin (HA) (Covance Research Products); anti-actin and anti-Flag (M2) (Sigma-Aldrich).

For confocal microscopy, rabbit anti-LAT (Upstate Biotechnology), mouse monoclonal anti-HA (Covance Research Products), goat anti-LAT (M-19), goat anti-HPK1 (N-19) (Santa Cruz Biotechnology), and rabbit anti-mouse Sprouty1 were used. Alexa-fluor 488 anti-rabbit, Alexa-fluor 546 anti-mouse, and Alexa-fluor 546 anti-goat Abs (Molecular Probe) were used as secondary reagents. Rabbit anti-mouse Sprouty1 Ab was generated by immunizing rabbits with histidine hexamer-tagged recombinant protein containing N-terminal region (amino acid residues 1–174) of mouse Sprouty1 (AbFrontier).

Plasmid constructs

The expression plasmid for HA-tagged Sprouty1 (pHM6-mSprouty1) was previously described (13). For the expression plasmid for Flag-tagged Sprouty1, Sprouty1 cDNA fragment isolated by digestion of pHM6-mSprouty1 with *Bam*HI and *Eco*RI was subcloned in-frame into pCMV-3Tag vector (Stratagene). The plasmid for Tet-off stable transfection was made by subcloning of Flag-Sprouty1 into pTRE-tight vector (Clontech). Deletion mutants of Sprouty1 were generated by PCR followed by subcloning into *Kpn*I and EcoR1 sites of HA-tag-containing expression vector (pHM6; Roche). Point mutants for tyrosine 53 and other tyrosine residues were generated using Quickchange site-directed mutagenesis kit (Stratagene). In some experiments, Flag-Sprouty1 and deletion mutants were cloned into EF1α promoter-driven expression vector (pcEFL, a gift from L. Samelson, National Cancer Institute/National Institutes of Health, Bethesda, MD) to assure efficient expression in T cells. All the sequences were confirmed by automated DNA sequencing.

Transient transfection

For luciferase assay, Jurkat T cells were washed twice with serum-free RPMI 1640 and $1.0–2.0 \times 10^7$ cells were resuspended in 500 μl of serum-free RPMI 1640 containing wild-type or mutant HA-Sprouty1 expression plasmid, human IL-2 promoter-driven firefly luciferase plasmid (a gift from S. Hoffmann, National Institute of Diabetes and Digestive and Kidney Diseases/National Institutes of Health, Bethesda, MD), and pRL-TK (Renilla luciferase control plasmid for normalization; Promega). Cells were electroporated at 250V and 950μF in a 0.4-cm-gap cuvette using Gene Pulser (Bio-Rad Laboratories) and allowed to recover for 24 h before stimulation. AP-1 luciferase plasmid (a gift from A. Weiss, University of California San Francisco, San Francisco, CA), NFAT luciferase plasmid, or NF-κB luciferase plasmid (gifts from D. McKean, Mayo College of Medicine, Rochester, MN) was used in place of human IL-2 luciferase plasmid in some experiments. For cotransfection and coimmunoprecipitation experiments, pcEFL-Flag-wild-type or deletion mutant Sprouty1 and pcEFL-myc-LAT (a gift from L. Samelson) plasmids were used in the above protocol.

Luciferase assay

For stimulation, a 96-well plate coated overnight with goat anti-mouse IgG (10 μg/ml) was washed with PBS twice and coated with anti-CD3 (1 μg/ml) for 2 h at room temperature. After washing three times with PBS, 1×10^5 cells were added to each well with soluble anti-CD28 (2 μg/ml) and incubated at 37°C for 6 h followed by lysis. Luciferase activity was measured with luminometer (VICTORlight; PerkinElmer) using dual-luciferase reporter assay system according to the manufacturer's instruction (Promega). Firefly luciferase activity was normalized with Renilla luciferase activity.

Stable transfection and IL-2 ELISA

Tet-off Jurkat T cells were electroporated with pTRE-tight-Flag-Sprouty1 plasmid and linear hygromycin marker DNA (Clontech) using Gene Pulser as detailed above. Forty-eight hours after transfection, 3×10^4 cells were seeded into each well of 24-well plates in a medium containing 100 μg/ml hygromycin, 100 μg/ml geneticin, and 2 μg/ml doxycycline for 2–3 wk. The selected clones were expanded for additional experiments. Expression of Sprouty1 proteins was induced by two consecutive washing of cells at 24-h interval, followed by 48-h culture without doxycycline. To measure IL-2 secreted into medium, cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 as described above, except that stimulation was allowed to proceed for 24 h. The amount of IL-2 in the culture supernatant was measured by OptEIA human IL-2 set (BD Biosciences) according to the manufacturer's instruction.

Confocal microscopy

For conjugation analysis using Jurkat cells, Raji B cells were stained with CellTracker Blue CMAC (Molecular Probes) and pulsed with or without 1 μg/ml staphylococcal enterotoxin E (SEE; Toxin Technologies) for 30 min at 37°C. Then, Raji B cells were mixed with an equal number of Jurkat T cells (4×10^5 cells) transfected with wild-type or mutant HA-Sprouty1 expression plasmids. For conjugation analysis using primary T cells, activated T cells were mixed with uncoated beads, anti-CD3, or anti-CD3/CD28 coated beads. After brief centrifugation and incubation for 15–20 min at 37°C, the cell mixture was gently resuspended, plated onto poly-L-lysine-precoated slide, and allowed to settle down for 15 min at room temperature. Cells were fixed for 20 min at room temperature with 3% formaldehyde in PBS and permeabilized for 5 min at room temperature with 0.3% Triton X-100 in PBS. Cells were then stained with indicated primary Abs and visualized by Alexa-Fluor 546- or Alexa-Fluor 488-labeled secondary Abs. For primary T cells, nuclei were stained with DAPI (Sigma-Aldrich) for 10 min at room temperature. Cover slips were mounted onto the slides with Prolong Gold Antifade reagent (Invitrogen) and fluorescent images were captured by confocal microscope (LSM 510 META; Carl Zeiss). Colocalization efficiency was calculated as the ratio of the number of colocalizing pixels to the total number of pixels above threshold in green channel (LAT for Jurkat cells) or red channel (LAT and HPK1 for primary T cells) using LSM 510 software (Carl Zeiss). Anti-CD3 or anti-CD3/CD28 coated beads were generated by coupling 50 μg of anti-mouse CD3 (or anti-mouse CD3 with 50 μg of anti-mouse CD28) to 1×10^8 magnetic beads (Dynabeads M450 Epoxy; Molecular Probes) according to manufacturer's instruction.

Immunoprecipitation and Western blot

Cells were washed twice with serum-free medium and resuspended at $4–10 \times 10^7$ cells/ml. Cells were then treated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) for 10 min on ice, followed by cross-linking with goat anti-mouse IgG (5 μg/ml) for 10 min on ice. For stimulation, cells were placed in a 37°C water bath for the indicated time and the reaction was stopped by adding ice-cold PBS. The cells were centrifuged and lysed with lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-Cl, pH7.5, 1 mM EDTA, pH 8.0, 20 mM NaF, 0.3 mM Na_3VO_4, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate) containing protease inhibitor mixture (Complete Mini; Roche). For immunoprecipitations, cell lysates were incubated with the indicated Abs for 3 h at 4°C, followed by incubation with 30 μl of protein G-agarose beads (Santa Cruz Biotechnology) for 1 h. Immune complexes were washed five times in lysis buffer before Western blotting. For some experiments, anti-Flag-conjugated agarose beads (Sigma-Aldrich) were used instead of protein G-agarose, in which case bound proteins were eluted by Flag-peptide (Sigma-Aldrich) before Western blotting. For Western blotting, immunoprecipitated proteins or total cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane (Millipore), and probed with the indicated Abs. HRP-conjugated secondary Abs (Jackson ImmunoResearch Laboratories) were used to detect primary Abs. Blots were visualized by chemiluminescence reaction using SuperSignal West Pico (Pierce). The band intensities were quantified with Kodak 1D software.

TAT-fusion protein transduction

Expression and purification of recombinant TAT-fusion proteins from the BL21 (DE3) pLysS strain of *E. coli* were performed as described (13). The final TAT-protein concentration was measured by SDS-PAGE in comparison with BSA standards and Coomassie blue staining. Naive T cells from 5C.C7 TCR-transgenic $Rag2^{-/-}$ mice were activated with plate-bound anti-mouse CD3ε (10 μg/ml) with soluble anti-mouse CD28 for 48 h. Cells were washed twice with PBS and incubated with TAT-proteins (200 nM) for 2 h in a CO_2 incubator. Then the cells were restimulated with various concentrations of plate-bound anti-CD3 plus anti-CD28 for 48 h. The amount of IL-2 protein in the culture supernatant was measured by OptEIA mouse IL-2 set (BD Biosciences) according to the manufacturer's instruction.

Results

Sprouty1 inhibits IL-2 transcription in Jurkat T cells

Previously, we showed Sprouty1 inhibits TCR signaling in murine $CD4^+$ T cell clones (13). To study molecular mechanism underlying this inhibition, we checked whether this inhibitory phenomenon could be reproduced in Jurkat T cell, a well-known human T cell line, which has been heavily used for understanding of TCR signaling (26). We cotransfected Sprouty1 expression plasmid with IL-2 promoter-driven luciferase plasmid into Jurkat cells and stimulated the transfectants with anti-CD3 plus anti-CD28, agonistic Abs for TCR signaling. As shown in Fig. 1*A*, overexpressed Sprouty1 markedly inhibited TCR signal-driven luciferase activity, reflecting IL-2 transcription, which mimicked the effect shown in T cell clones. When TCR becomes engaged with Ag, a series of proximal signaling molecules are activated in cascade, promoting IL-2 transcription via three major pathways: calcium-NFAT pathway, NF-κB pathway, and Ras-MAPK-AP-1 pathway. To determine which of these three pathways are affected by Sprouty1 overexpression, Sprouty1 plasmid was cotransfected with reporter plasmids containing the responsive elements to NFAT, NF-κB, and AP-1. Sprouty1 inhibited all three reporter activities driven by TCR signal, implying that it may work at the proximal level before splitting into these pathways (Fig. 1, *B–D*).

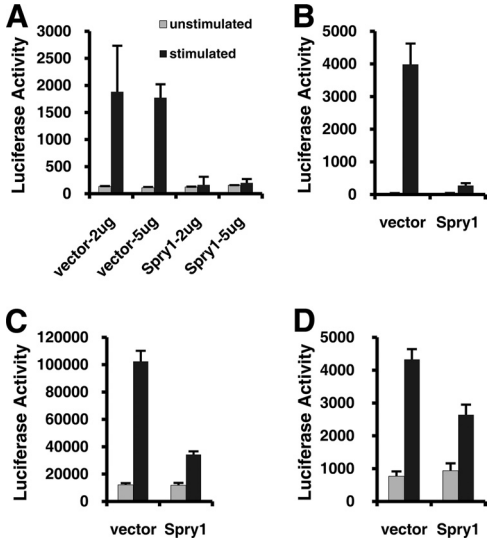

FIGURE 1. Sprouty1 (Spry1) inhibits TCR signal-driven IL-2 transcription in Jurkat cells. Jurkat T cells were transfected with 20 μg IL-2-promoter luciferase plasmid (*A*), 10 μg NFAT luciferase plasmid (*B*), 20 μg AP-1 luciferase plasmid (*C*), or 10 μg NF-κB luciferase plasmid (*D*) along with empty vector or Sprouty1 expression plasmid (5 μg; *B–D*) and 0.5 μg of TK-Renilla luciferase plasmid. After 24 h, the cells were stimulated with anti-CD3 and anti-CD28 Abs for 6 h and firefly luciferase activity was measured from cell lysates. Transfection efficiency was normalized with Renilla luciferase activity.

Sprouty1 interacts with proximal signaling molecules of TCR signaling

To study the details of the molecular events involved in this inhibitory phenomenon, we generated an inducible stable transfectant of Jurkat cells using Tet-off system, in which Sprouty1 is induced by removal of tetracycline or doxycycline. When doxycycline was removed from the culture supernatant, Sprouty1 protein induction was detected after 72 h (Fig. 2*A*). Inhibitory effect of Sprouty1 on IL-2 production was further evaluated by measuring the amount of IL-2 protein secreted into culture supernatant after stimulation with anti-CD3 plus anti-CD28. As expected, the amount of secreted IL-2 profoundly decreased when Sprouty1 was induced compared with that of the un-induced condition, confirming the result obtained from IL-2 promoter-driven luciferase activity assay (Fig. 2*B*).

To determine with which proteins Sprouty1 interacts, we immunoprecipitated Sprouty1 and tried to identify coimmunoprecipitated proteins by Western blot. Because many of the signaling molecules become phosphorylated at tyrosine residues after TCR

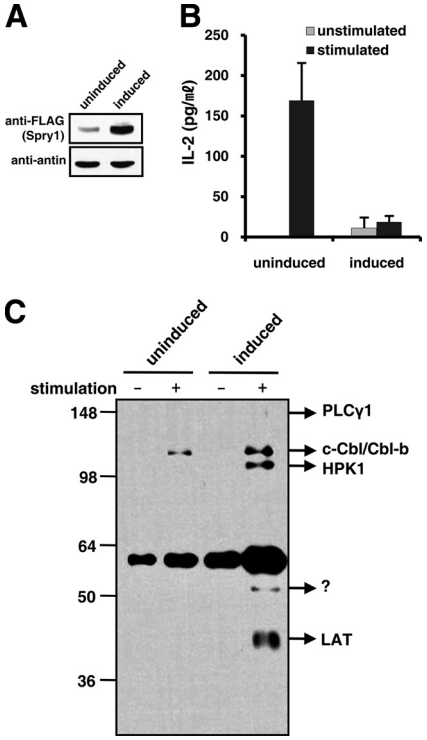

FIGURE 2. Sprouty1 (Spry1) interacts with tyrosine-phosphorylated proteins upon TCR stimulation in a stable transfectant of Jurkat cells. FLAG-Sprouty1-expressing Tet-off Jurkat cells were cultured in presence or absence of doxycycline for 72 h. *A*, Cell lysates were immunoblotted with anti-FLAG M2 mAb or anti-actin as a loading control. *B*, Cells were stimulated with plate-bound anti-CD3 plus anti-CD28 for 24 h and the amount of IL-2 in the culture supernatant was measured by ELISA. *C*, After stimulating 5×10^7 cells with soluble anti-CD3 plus anti-CD28 for 2 min, cell lysates were immunoprecipitated with anti-FLAG M2 mAb and immunoblotted with anti-phosphotyrosine Ab.

stimulation, we immunoprecipitated Sprouty1 and performed Western blot with anti-phosphotyrosine Ab. Five tyrosine-phosphorylated bands were identified when stimulated with anti-CD3 plus anti-CD28 (Fig. 2*C*). Based on their molecular size, LAT, PLC-γ1, c-Cbl/Cbl-b, and HPK1 appeared to be candidate molecules for interaction with Sprouty1. Interaction with each of these molecule was confirmed by immunoprecipitation with Ab to Flag-Sprouty1 and Western blot with Abs to candidate molecules (Fig.

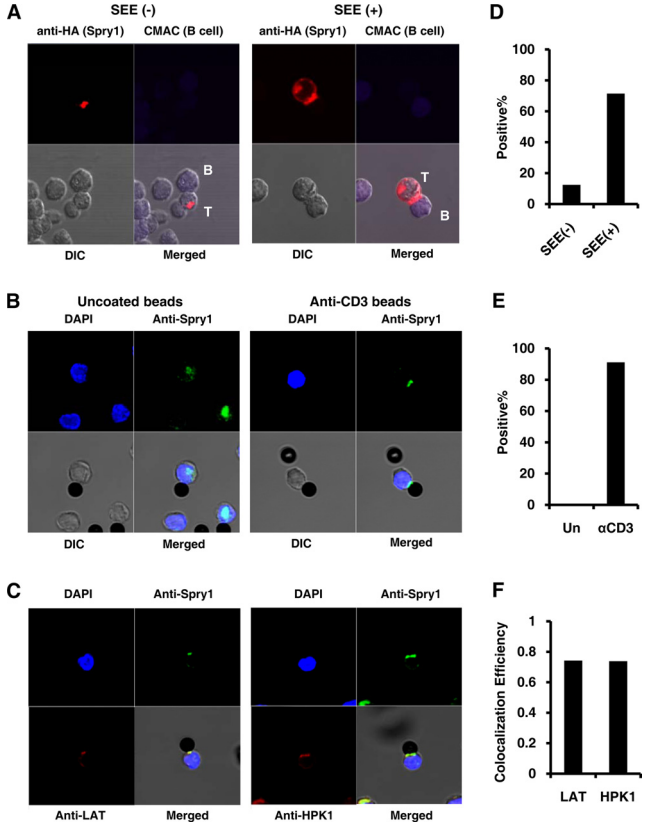

FIGURE 4. Sprouty1 (Spry1) translocates to T cell-APC contact site upon antigenic stimulation. *A*, Jurkat T cells were transiently transfected with HA-Sprouty1 expression plasmid. After 24 h, CMAC (blue)-loaded Raji B cells were allowed to conjugate with Jurkat T cells in the absence or presence of SEE on poly-L-lysine-coated cover slips. Cells were fixed, permeabilized, and stained with anti-HA Ab, followed by Alexa Fluor 546-labeled secondary Ab (red). *B* and *C*, 24-h-activated primary murine $CD4^+$ T cells were allowed to conjugate with uncoated beads, anti-CD3- (*B*), or anti-CD3/CD28-coated beads (*C*). Fixed and permeabilized cells were stained with anti-Sprouty1 Ab either alone (*B*) or with anti-LAT or anti-HPK1 (*C*). Alexa Fluor 488- and 546-labeled secondary Abs were used for detection. Cell conjugates were visualized with confocal microscopy. *D–F*, Histograms depict the percentage of T cells showing a clear localization of Sprouty1 in immune synapse for Jurkat cells (*D*) and primary T cells (*E*) or the median value of colocalization efficiency between Sprouty1 and LAT/HPK1 (*F*) in anti-CD3/CD28 bead-conjugated primary T cells. Quantification was performed on more than 50 conjugates for each histogram. T, Jurkat T cells; B, Raji B cells; DIC, differential interference contrast.

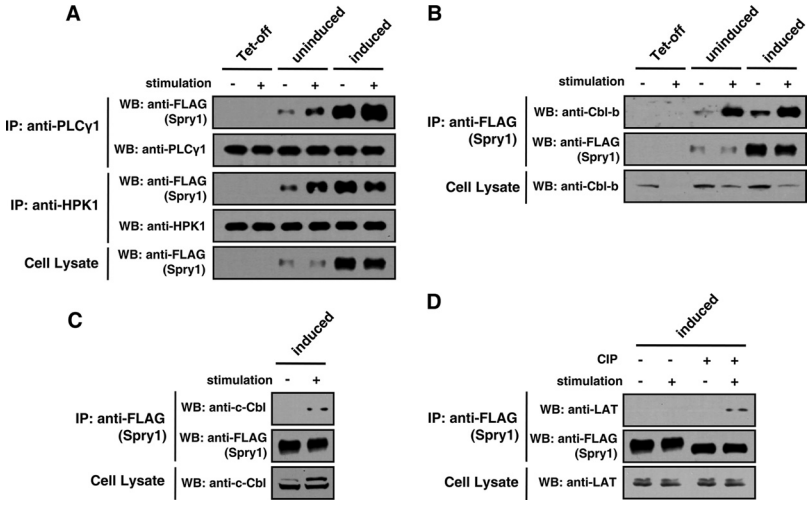

FIGURE 3. Sprouty1 (Spry1) interacts with proximal TCR signaling molecules upon TCR stimulation. Tet-off Jurkat cells or FLAG-Sprouty1-expressing Tet-off Jurkat cells were cultured in the presence or absence of doxycycline for 72 h. Cells were stimulated with soluble anti-CD3 plus anti-CD28 for 2 min before lysis. *A*, Cell lysates were immunoprecipitated (IP) with anti-PLC-γ1 or anti-HPK1, and immunoblotted with anti-FLAG M2. *B–D*, Cell lysates were immunoprecipitated with anti-FLAG M2, followed by Western blotting (WB) with anti-Cbl-b (*B*), anti-c-Cbl (*C*), and anti-LAT (*D*). For *D*, immunoprecipitated proteins were treated with 50 U of calf intestinal phosphatase (CIP; New England Biolabs) for 1 h at 37°C before Western blotting.

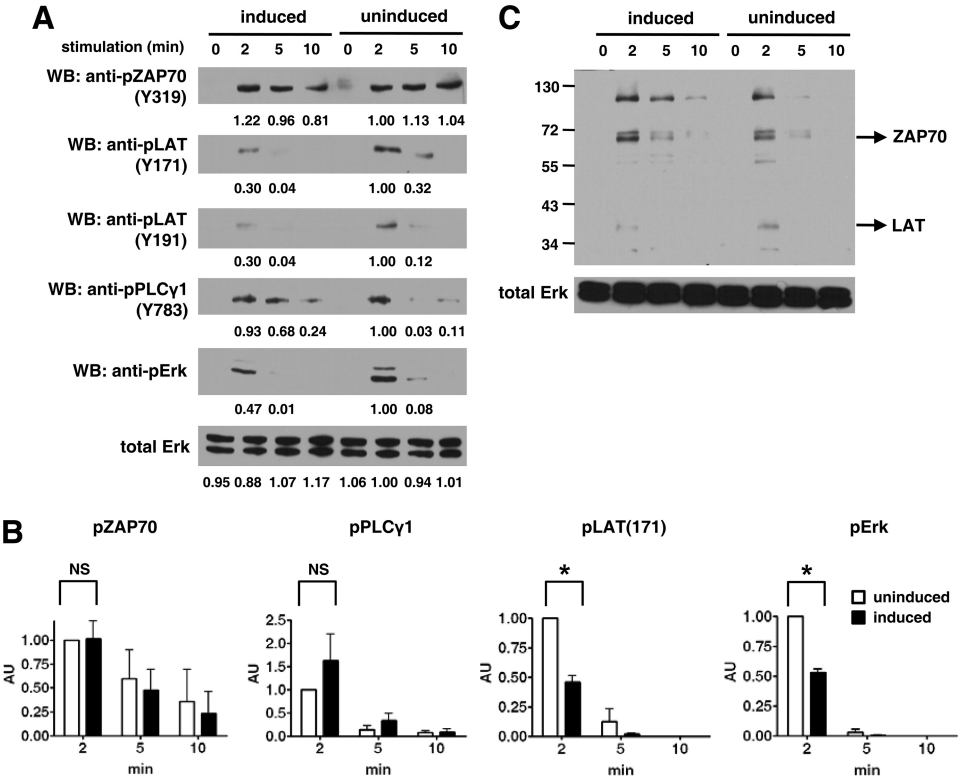

FIGURE 5. Sprouty1 inhibits TCR stimulus-driven LAT phosphorylation. Tet-off Jurkat cells or FLAG-Sprouty1-expressing Tet-off Jurkat cells were cultured in the presence or absence of doxycycline for 72 h. Cells were stimulated with soluble anti-CD3 plus anti-CD28 for 2, 5, and 10 min before lysis. *A* and *B*, Cell lysates were subjected to Western blotting (WB) with indicated Abs (*A*). Anti-total Erk was used to prove equal loading. The values below each blot represent the relative band intensities. The quantified data from three independent experiments are shown as graphs (*B*). The arbitrary unit (AU) represents the normalized value with total Erk intensity. Results were analyzed by paired Student's *t* test. NS, Not significant; ∗, $p < 0.02$. *C*, Cell lysates were immunoblotted with anti-phosphotyrosine Ab. Anti-total Erk was used to prove equal loading.

3, *B–D*) or vice versa (Fig. 3*A*). PLC-γ1, HPK1, and Cbl-b interacted with Sprouty1 in the absence of stimulation, whereas interaction between Sprouty1 and c-Cbl or LAT was only detected after TCR stimulation. However, all of these interactions were potentiated by anti-CD3 plus anti-CD28 stimulus, suggesting that TCR signaling may affect functional interaction of Sprouty1 with target proteins. Because anti-LAT Ab does not interact with phosphorylated LAT (27), interaction with LAT was detected by anti-LAT Ab only after phosphatase treatment of immunoprecipitates (Fig. 3*D*).

Sprouty1 translocates to immune synapse upon Ag engagement

The facts that all of the Sprouty1-interacting proteins are components of proximal TCR signalosome and that these interactions are potentiated by TCR stimulation indicate that Sprouty1 might be recruited to proximal signaling complex upon TCR ligation and play a role in the negative regulation of signaling. The proximal signaling molecules, such as LAT and HPK1, have been known to localize to T cell-APC contact site, so-called immune synapse upon Ag engagement. Therefore, we examined whether Sprouty1 localizes to immune synapse upon Ag engagement, using SEE as a model Ag. When Jurkat cells were transiently transfected with HA-tagged Sprouty1 and incubated with Raji B cells as APCs, Sprouty1 mainly localized to the cytosol. However, when these T cells were incubated with SEE-loaded Raji cells, Sprouty1 was localized to T cell-B cell contact site, where it forms cell conjugates (Fig. 4, *A* and *D*), supporting the idea of its participation in proximal signaling complex. These observations were extended to the case of activated primary murine $CD4^+$ T cells, where endogenous Sprouty1 proteins localized to immune synapse when primary T cells were incubated with anti-CD3-coated beads as artificial APCs, but not when incubated with uncoated beads (Fig. 4, *B* and *E*). Furthermore, translocated Sprouty1 colocalized with endogenous LAT and HPK1, in agreement with coimmunoprecipitation data obtained with Jurkat cells (Fig. 4, *C* and *F*).

Sprouty1 inhibits TCR stimulus-induced LAT phosphorylation

Next, we tried to pinpoint the site where Sprouty1 acts to inhibit TCR signaling. Sprouty1-induced transfectants of Jurkat cells were stimulated with anti-CD3 plus anti-C28 and the degree of phosphorylation of each signaling molecules was compared with that of un-induced cells. As expected from an earlier finding (13), phosphorylation of Erk1/2 was remarkably reduced when Sprouty1 was induced (Fig. 5, *A* and *B*). Upstream phosphorylation of ZAP70 was not affected by Sprouty1 overexpression. In contrast, LAT phosphorylation at tyrosine 171 and 191 clearly decreased when Sprouty1 was induced. This pattern was also confirmed by Western blot with anti-phosphotyrosine Ab, in which phosphorylation of a 36kD band, presumably LAT, decreased by Sprouty1 induction, whereas phosphorylation of a 70kD band, presumably ZAP70, did not (Fig. 5*C*). Interestingly, phosphorylation of downstream PLC-γ1 was not affected by Sprouty1 induction (Fig. 5, *A* and *B*), nor was calcium influx affected (data not shown). Collectively, these findings suggest that Sprouty1 inhibits TCR-driven LAT activation, leading to impaired activation of Erk and IL-2 production.

C-terminal region of Sprouty1 is essential for its inhibitory activity

To see which domain of Sprouty1 contributes to its inhibitory activity, we generated several deletion mutants. Sprouty1 has a conserved C-terminal half (cysteine-rich domain), and a less-conserved N-terminal half. When the mutant containing only the N-terminal half was cotransfected with IL-2 promoter-driven luciferase plasmid to Jurkat cells that were stimulated with anti-CD3 plus CD28, it did not show any inhibitory activity, whereas the mutant with only the C-terminal half showed inhibitory effect comparable to the wild-type Sprouty1, indicating that the C-terminal half is responsible for the inhibitory activity of Sprouty1 (Fig. 6*A*). Because phosphorylated tyrosine 55 of Sprouty2 (tyrosine 53 in

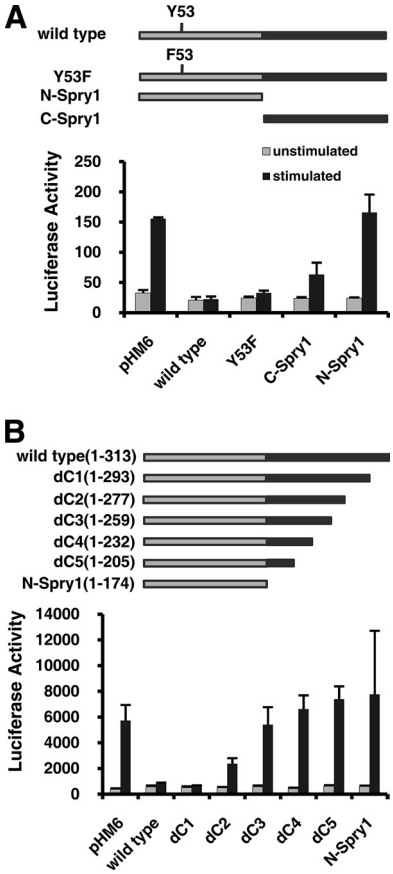

FIGURE 6. C-terminal region of cysteine-rich domain (259–293) is essential for Sprouty1 action. Jurkat T cells were transfected with 20 μg IL-2-promoter luciferase plasmid along with 5 μg empty vector. wild-type, or mutant HA-Sprouty1 expression plasmid and 0.5 μg of TK-Renilla luciferase plasmid. After 24 h, the cells were stimulated with anti-CD3 and anti-CD28 Abs for 6 h and firefly luciferase activity was measured from cell lysates. Transfection efficiency was normalized with Renilla luciferase activity. *A*, Y53F, Sprouty1 with tyrosine 53 replaced by phenylalanine; N-Spry1, N-terminal half of Sprouty1; C-Spry1, C-terminal half of Sprouty1. *B*, dC1–5, serial deletion mutants of C terminus of Sprouty1.

Sprouty1) was shown to interact with Grb2, causing inhibition of FGFR signaling and its substitution by phenylalanine abrogated the inhibitory activity, we checked whether Y53F mutant of Sprouty1 loses inhibitory activity on IL-2 transcription. However, this mutant inhibited IL-2 transcription to the same degree as the wild-type Sprouty1, which is in line with our finding with the C-terminal half-mutant (Fig. 6*A*). To narrow down the responsible motif in C-terminal half, we generated serial C-terminal deletion mutants and performed cotransfection as described above. Deletion of C-terminal 54 amino acids (dC3) completely abolished the inhibitory effect of Sprouty1, suggesting that this region may play a critical role in Sprouty1 function (Fig. 6*B*).

C-terminal 54 amino acids are critical for colocalization and interaction with LAT

Noting that Sprouty1 might inhibit phosphorylation of LAT through translocation to immune synapse upon Ag engagement and C-terminal 54 amino acids was critical for its functional activity, we attempted to see whether this region contributes to colocalization of Sprouty1 and LAT upon Ag engagement. Jurkat cells transiently transfected with C-terminal deletion mutants of Sprouty1 were incubated with SEE-loaded Raji cells and stained with fluorescent-labeled Abs specific to each molecule. As expected, the wild-type Sprouty1 and the mutant lacking C-terminal 20 amino acids (dC1) colocalized with LAT at immune synapse upon Ag engagement. In contrast, dC3 and dC5 were not able to colocalize with LAT under the same conditions (Fig. 7*A*). This phenomenon was quantified by counting the cell conjugates showing costaining (Fig. 7*B*) and by calculating colocalization efficiency of the cell conjugates (Fig. 7*C*). These data reveal that deletion of 54 amino acids abolishes the interaction between the two molecules. Accordingly, when dC3 mutant was cotransfected with LAT, the mutant was coimmunoprecipitated with LAT to a far less degree than the wild-type Sprouty1 (Fig. 7*D*). Thus, Sprouty1 uses C-terminal 54 amino acids to translocate to immune synapse and interact with LAT.

C-terminal 54 amino acids are essential for Sprouty1 action in primary T cells

Finally, we examined whether C-terminal deletion of 54 amino acids would abolish the inhibitory effect of Sprouty1 on activated primary $CD4^+$ T cells. Recombinant wild-type and dC3 mutant proteins were purified as cell-permeable TAT-fusion proteins for overexpression in primary T cells. This strategy was successfully used in a previous study (13). We transduced TAT-fusion proteins to 48-h-activated naive $CD4^+$ T cells from TCR-transgenic, $Rag2^{-/-}$ mice and reactivated them to see whether overexpression of Sprouty1 would inhibit further activation of T cells. TAT-GFP protein was used as a negative control. The wild-type Sprouty1 inhibited TCR-signal induced IL-2 production, whereas dC3 mutant was not able to inhibit IL-2 production (Fig. 8). Thus, Sprouty1 seems to serve as a negative regulator to prevent further activation of activated T cells and the C-terminal region of Sprouty1 is essential for this activity.

Discussion

TCR signaling accompanies several negative regulation machineries in addition to the positive signals. Some of them become activated immediately after TCR signal begins to regulate excessive activation of T cells, whereas others become induced at a delayed time point to dampen down T cell response after the T cells fulfill an appropriate mission. The immediate negative regulators are mostly intracellular signaling molecules, such as phosphatases (Shp1, STS2), kinases (Csk, HPK1), and adaptors (PAG, DOK1/2) (28). The delayed negative regulators are usually surface receptors and cytokines, as exemplified in the introduction section. Sprouty1, as an intracellular signaling adaptor, is quite exceptional, as it is induced at a later time point when delayed extracellular regulators work. This may provide an additional mechanism for cell-intrinsic refractoriness to further T cell activation.

The inhibitory effect of Sprouty1 in T cells was demonstrated by both gain-of-function and loss-of-function studies in our previous report (13). Overexpression of Sprouty1 inhibited TCR signal-driven IL-2 production and Erk activation, whereas small interfering RNA-mediated knockdown of Sprouty1 enhanced IL-2 production in murine T cell clones. However, the use of T cell clones for understanding the molecular mechanism was hampered by the difficulty of maintaining T cell clones and the low yields of TAT-Sprouty1 fusion proteins used in overexpression studies. Jurkat T cells are a continuously cycling T cell line and TCR signaling is relatively well preserved. Therefore, they may represent actively proliferating T cells. Coherently, TAT-Sprouty1 treatment of actively proliferating primary T cells inhibited further production of IL-2 by TCR stimulus (Fig. 8).

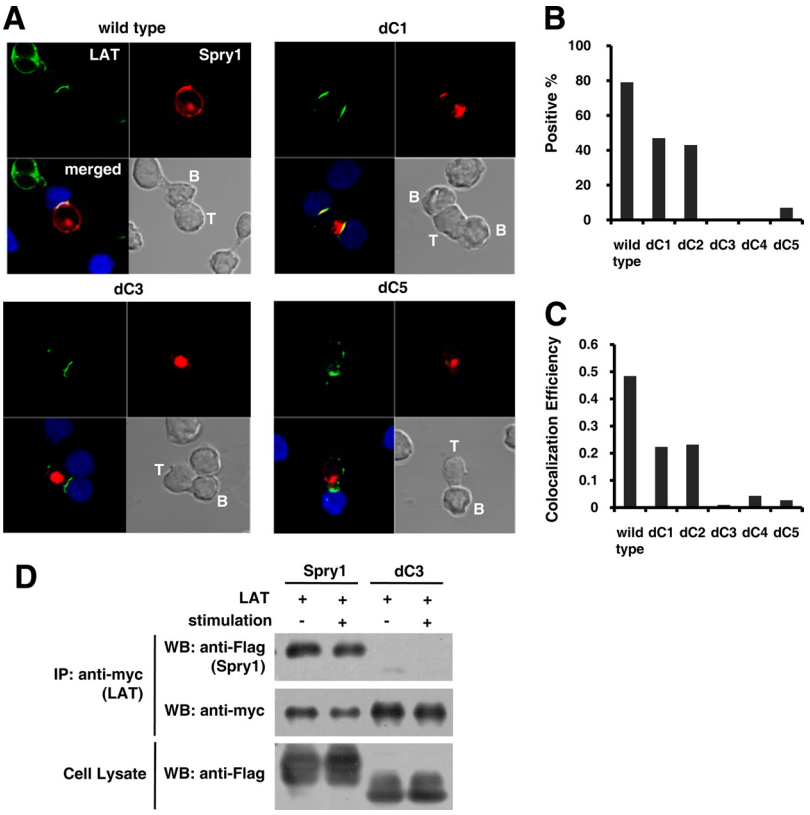

FIGURE 7. C-terminal 54 amino acids of Sprouty1 (Spry1) are important for its colocalization and interaction with LAT in immune synapse. *A*, Jurkat T cells were transfected with wild-type or deletion mutant HA-Sprouty1 plasmid. After 24 h, transfectants were conjugated with CMAC (blue)-labeled Raji B cells in the presence of SEE and transferred to poly-L-lysine-coated cover slip. Cells were fixed, permeabilized, and stained with anti-HA and anti-LAT Abs. The secondary Ab for HA-Sprouty1 was Alexa-Fluor 546 labeled anti-mouse Ab (red) and Ab for LAT was Alexa-Fluor 488 labeled anti-rabbit Ab (green). Cell conjugates were visualized with confocal microscopy. *B* and *C*, Histograms depict the percentage of T cells showing a clear colocalization between Sprouty1 and LAT (*B*) or the median value of colocalization efficiency (*C*). Quantification was performed on more than 25 conjugates for each construct. *D*, FLAG-tagged wild-type or deletion mutant Sprouty1 plasmid and myc-tagged LAT plasmid were cotransfected into Jurkat T cells. After 24 h, cell lysates were immunoprecipitated with anti-myc Ab and immunoblotted with anti-FLAG M2 Ab. WB, Western blot; IP, immunoprecipitated. T, Jurkat T cells; B, Raji B cells.

Using Jurkat T cells, we could reproduce the inhibitory effect of Sprouty1 on IL-2 production induced by TCR signal at the transcriptional level using luciferase reporter assay. This effect was further confirmed at the protein level by ELISA in Sprouty1-stable transfectants. Sprouty1 overexpression also inhibited AP-1, NFAT-specific reporter activities, which correlated with reduced Erk and NFAT activation observed in T cell clones. Sprouty1 was reported to work at Ras-MAPK pathway exclusively in FGFR signaling in epithelial cells without affecting other pathways, such as PI3K, JNK, and p38 (17, 22). However, in TCR signaling, Sprouty1 affects multiple pathways simultaneously, including Ras-MAPK, NFAT, and NF-κB, which led to the assumption that it works at the proximal signalosome before branching into these specific pathways. As expected, Sprouty1 binds to major proteins of TCR signalosome, such as LAT, PLC-γ1, c-Cbl, Cbl-b, and HPK1. However, we could not detect tyrosine-phosphorylated ZAP70 in Sprouty1 immunoprecipitates, which lies upstream of LAT (Fig. 2*C*). Accordingly, Sprouty1 inhibited LAT phosphorylation following TCR stimulus without affecting upstream ZAP70 phosphorylation. Therefore, Sprouty1 seems to operate at the level of LAT in TCR signaling.

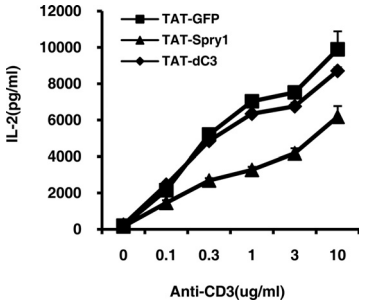

FIGURE 8. C-terminal 54 amino acids are essential for Sprouty1 (Spry1) action in primary T cells. Naive $CD4^+$ T cells from TCR-transgenic, $Rag2^{-/-}$ mice were activated with anti-CD3 plus anti-CD28. After 48 h, T cells were transduced with TAT-proteins for 2 h, followed by restimulation with various concentrations of anti-CD3 plus anti-CD28 for 48 h. The amount of secreted IL-2 was measured by ELISA from the culture supernatant.

It is not clear at this point how Sprouty1 inhibits LAT phosphorylation. One possibility would be that direct binding of Sprouty1 with LAT prevents LAT from being phosphorylated. However, Sprouty1 does not have SH2 domain needed for interaction with phosphotyrosines of LAT, which provide major binding sites with other interacting molecules including SLP76, Grb2, GADS, and PLC-γ1. Another possibility is that Sprouty1 recruits inhibitory molecules around LAT and provides a bridge between them. C-Cbl or Cbl-b could be one of those inhibitory molecules. Sprouty2 is known to bind to c-Cbl constitutively or inducibly in EGF receptor (EGFR) signaling (29, 30), although in that case, Sprouty2 sequestered c-Cbl from EGFR, resulting in enhanced EGFR signaling. In T cells, c-Cbl and Cbl-b exert negative effect on TCR signaling by ubiquitinating various signaling molecules, such as CD3ζ-chain of TCR complex (31), ZAP70 (32, 33), Lck (34), Fyn (35), Vav (36), PI3K (37), and, importantly, LAT (38). Therefore, Sprouty1 might recruit c-Cbl and Cbl-b to LAT complex, thereby promoting ubiquitination and degradation of LAT. Another inhibitory molecule that binds to Sprouty1 is HPK1. Overexpression of HPK1 in Jurkat T cells inhibits activation of Erk and AP-1 transcription factor (39) and small interfering RNA knockdown of HPK1 in Jurkat cells (40) or genetic knockout of HPK1 in mice (41) enhances TCR signaling. Recently, molecular mechanism of this inhibition was partially explained by that HPK1 phosphorylates serine 364 of SLP76, to which 14-3-3, a known negative regulator, binds (42). Thus, it is also possible that Sprouty1 facilitates recruitment of HPK1 to the proximity of LAT, further enhancing its inhibitory activity. Interestingly, 14-3-3 is also known to bind to c-Cbl upon TCR signaling (43). Functional

relationship between Sprouty1 and Cbl proteins and/or HPK1 is now under investigation.

The finding that phosphorylation of PLC-γ1 was not affected by Sprouty1 overexpression despite reduced LAT phosphorylation is intriguing. One explanation is that calcium pathway is more sensitive to TCR signal than Erk pathway, so that low amounts of phosphorylated LAT are enough to trigger PLC-γ1 phosphorylation. In contrast, reduced LAT phosphorylation might be enough to cause decreased Erk phosphorylation and IL-2 production. It is known that Erk can be activated by two routes. In one, phosphorylated LAT binds to Grb2-SOS, which activates Ras directly (44). In another, PLC-γ1 activation followed by LAT phosphorylation can produce diacylglycerol, an activator of RasGRP, which activates Ras (45, 46). Therefore, reduced Erk activation caused by Sprouty1 induction in a stable transfectant can result from inhibition of LAT-Grb2-SOS-RAS pathway. Nonetheless, NFAT reporter activity, which need IP_3 produced by PLC-γ1 for activation, decreased when Sprouty1 was transiently overexpressed. Consistent with this observation, NFAT dephosphorylation (activation) was reduced in the stable transfectant (data not shown). However, calcium flux after TCR stimulation in the stable transfectant was not decreased, meaning that Sprouty1 may have an additional action site immediately before NFAT activation step, presumably at the level of calcineurin. Accordingly, we recently observed that Sprouty1 interacts with a calcineurin inhibitor, Cabin1 (J. S. Lee and K. Choi, unpublished results). Thus, Sprouty1 may have multiple action sites to control T cell activation.

Proximal signaling molecules of TCR signalosome, such as LAT, c-Cbl, and HPK1, are recruited to T cell-APC contact (immune synapse) site (38, 40, 47). Interaction of Sprouty1 with these molecules led us to examine whether Sprouty1 is recruited to immune synapse. As expected, upon Ag engagement, Sprouty1 translocated from cytosol to T cell-APC contact site. This activation-induced recruitment correlated with inducible interaction of Sprouty1 with these molecules biochemically. Importantly, Sprouty1 and LAT colocalization was abolished in the case of functionally defective mutants of Sprouty1, further confirming the importance of Sprouty1 translocation in Sprouty1 function.

Structural importance of domains of Sprouty1 was evaluated using a series of deletion mutants. Interestingly, we found that the C-terminal half of Sprouty1 was sufficient to inhibit IL-2 transcription. This finding was quite different from the report on FGFR signaling that N-terminal tyrosine phosphorylation at 55 of Sprouty2 (53 for Sprouty1) was critical for inhibitory function (20, 21). This phosphorylated tyrosine was known to bind to SH2 domain of Grb2 in FGFR signaling (21) and to c-Cbl in EGFR signaling (30). In our system, mutation of tyrosine 53 to phenylalanine had no effect on Sprouty1 activity, supporting our conclusions from the deletion mutant data. We generated point mutants for each tyrosine residue in Sprouty1 and none of the mutants lost inhibitory activity, which shows that tyrosine phosphorylation of Sprouty1 is not important for Sprouty1 action in Jurkat cells (data not shown). Instead, it resembles Sprouty4 in VEGF signaling, in that the N-terminal tyrosine was not important and the C-terminal half was enough to exert negative effect (24). To further identify the essential functional motifs, we generated and studied serial deletion mutants of C terminus and found that 54 amino acids are important to Sprouty1 function. This region partially overlaps with the region reported to be important for its translocation to membrane in response to FGF (21). Accordingly, this deletion mutant was not able to translocate to immune synapse following TCR stimulus.

Collectively, the whole sequence of negative regulation of TCR signaling by Sprouty1 can be summarized as follows. Sprouty1, which is induced by TCR stimulus, may accumulate in the cytosol, waiting for the next stimulus. Upon re-engagement of TCR by Ag, Sprouty1 is recruited to the immune synapse by the C terminus translocation motif and interacts with proximal signaling molecules, including LAT, to inhibit downstream signaling. Although several other intracellular adaptor proteins have been reported to work on TCR signalosome, Sprouty1 adds temporal control to the spatial regulation of TCR signaling. In summary, we identified Sprouty1 as a negative feedback regulator of TCR signaling operating at the level of LAT. The physiological relevance of this biochemical information could be addressed in the future using genetically engineered mice such as knockout mouse or dominant-negative transgenic mouse, which may show enhanced T cell reactivity or autoimmune phenotype. Furthermore, genetic manipulation of Sprouty1 in T cells may enhance activation of tumor-reactive T cells, contributing to antitumor immunity.

Acknowledgments

We thank Mi Ae Kim for assistance in confocal microscopy.

Disclosures

The authors have no financial conflict of interest.

References

1. Van Parijs, L., and A. K. Abbas. 1998. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science* 280: 243–248.
2. Lenardo, M., K. M. Chan, F. Hornung, H. McFarland, R. Siegel, J. Wang, and L. Zheng. 1999. Mature T lymphocyte apoptosis: immune regulation in a dynamic and unpredictable antigenic environment. *Annu. Rev. Immunol.* 17: 221–253.
3. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1: 405–413.
4. Agata, Y., A. Kawasaki, H. Nishimura, Y. Ishida, T. Tsubata, H. Yagita, and T. Honjo. 1996. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int. Immunol.* 8: 765–772.
5. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 19: 683–765.
6. Li, M. O., and R. A. Flavell. 2008. TGF-β: a master of all T cell trades. *Cell* 134: 392–404.
7. Nishimura, H., T. Okazaki, Y. Tanaka, K. Nakatani, M. Hara, A. Matsumori, S. Sasayama, A. Mizoguchi, H. Hiai, N. Minato, and T. Honjo. 2001. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 291: 319–322.
8. Takahashi, T., M. Tanaka, C. I. Brannan, N. A. Jenkins, N. G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* 76: 969–976.
9. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 270: 985–988.
10. Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 11: 141–151.
11. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75: 263–274.
12. Kulkarni, A. B., C. G. Huh, D. Becker, A. Geiser, M. Lyght, K. C. Flanders, A. B. Roberts, M. B. Sporn, J. M. Ward, and S. Karlsson. 1993. Transforming growth factor β_1 null mutation in mice causes excessive inflammatory response and early death. *Proc. Natl. Acad. Sci. USA* 90: 770–774.
13. Choi, H., S. Y. Cho, R. H. Schwartz, and K. Choi. 2006. Dual effects of Sprouty1 on TCR signaling depending on the differentiation state of the T cell. *J. Immunol.* 176: 6034–6045.
14. Hacohen, N., S. Kramer, D. Sutherland, Y. Hiromi, and M. A. Krasnow. 1998. *sprouty* encodes a novel antagonist of FGF signaling that patterns apical branching of the *Drosophila* airways. *Cell* 92: 253–263.
15. Casci, T., J. Vinos, and M. Freeman. 1999. Sprouty, an intracellular inhibitor of Ras signaling. *Cell* 96: 655–665.
16. Reich, A., A. Sapir, and B. Shilo. 1999. Sprouty is a general inhibitor of receptor tyrosine kinase signaling. *Development* 126: 4139–4147.
17. Gross, I., B. Bassit, M. Benezra, and J. D. Licht. 2001. Mammalian sprouty proteins inhibit cell growth and differentiation by preventing ras activation. *J. Biol. Chem.* 276: 46460–46468.
18. Lim, J., P. Yusoff, E. S. Wong, S. Chandramouli, D. H. Lao, C. W. Fong, and G. R. Guy. 2002. The cysteine-rich Sprouty translocation domain targets mitogen-activated protein kinase inhibitory proteins to phosphatidylinositol 4,5-bisphosphate in plasma membranes. *Mol. Cell Biol.* 22: 7953–7966.
19. Tefft, D., M. Lee, S. Smith, D. L. Crowe, S. Bellusci, and D. Warburton. 2002. mSprouty2 inhibits FGF10-activated MAP kinase by differentially binding to

upstream target proteins. *Am. J. Physiol. Lung Cell Mol. Physiol.* 283: L700–L706.
20. Sasaki, A., T. Taketomi, T. Wakioka, R. Kato, and A. Yoshimura. 2001. Identification of a dominant negative mutant of Sprouty that potentiates fibroblast growth factor- but not epidermal growth factor-induced ERK activation. *J. Biol. Chem.* 276: 36804–36808.
21. Hanafusa, H., S. Torii, T. Yasunaga, and E. Nishida. 2002. Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. *Nat. Cell Biol.* 4: 850–858.
22. Yusoff, P., D. H. Lao, S. H. Ong, E. S. Wong, J. Lim, T. L. Lo, H. F. Leong, C. W. Fong, and G. R. Guy. 2002. Sprouty2 inhibits the Ras/MAP kinase pathway by inhibiting the activation of Raf. *J. Biol. Chem.* 277: 3195–3201.
23. Rubin, C., Y. Zwang, N. Vaisman, D. Ron, and Y. Yarden. 2005. Phosphorylation of carboxyl-terminal tyrosines modulates the specificity of Sprouty-2 inhibition of different signaling pathways. *J. Biol. Chem.* 280: 9735–9744.
24. Sasaki, A., T. Taketomi, R. Kato, K. Saeki, A. Nonami, M. Sasaki, M. Kuriyama, N. Saito, M. Shibuya, and A. Yoshimura. 2003. Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. *Nat. Cell Biol.* 5: 427–432.
25. Lin, J., and A. Weiss. 2001. T cell receptor signalling. *J. Cell Sci.* 114: 243–244.
26. Abraham, R. T., and A. Weiss. 2004. Jurkat T cells and development of the T-cell receptor signalling paradigm. *Nat. Rev. Immunol.* 4: 301–308.
27. Zhang, W., J. Sloan-Lancaster, J. Kitchen, R. P. Trible, and L. E. Samelson. 1998. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* 92: 83–92.
28. Acuto, O., V. D. Bartolo, and F. Michel. 2008. Tailoring T-cell receptor signals by proximal negative feedback mechanisms. *Nat. Rev. Immunol.* 8: 699–712.
29. Wong, E. S., J. Lim, B. C. Low, Q. Chen, and G. R. Guy. 2001. Evidence for direct interaction between Sprouty and Cbl. *J. Biol. Chem.* 276: 5866–5875.
30. Fong, C. W., H. F. Leong, E. S. Wong, J. Lim, P. Yusoff, and G. R. Guy. 2003. Tyrosine phosphorylation of Sprouty2 enhances its interaction with c-Cbl and is crucial for its function. *J. Biol. Chem.* 278: 33456–33464.
31. Wang, H. Y., Y. Altman, D. Fang, C. Elly, Y. Dai, Y. Shao, and Y. C. Liu. 2001. Cbl promotes ubiquitination of the T cell receptor zeta through an adaptor function of Zap-70. *J. Biol. Chem.* 276: 26004–26011.
32. Lupher, M. L., Jr., K. A. Reedquist, S. Miyake, W. Y. Langdon, and H. Band. 1996. A novel phosphotyrosine-binding domain in the N-terminal transforming region of Cbl interacts directly and selectively with ZAP-70 in T cells. *J. Biol. Chem.* 271: 24063–24068.
33. Rao, N., M. L. Lupher, Jr., S. Ota, K. A. Reedquist, B. J. Druker, and H. Band. 2000. The linker phosphorylation site Tyr292 mediates the negative regulatory effect of Cbl on ZAP-70 in T cells. *J. Immunol.* 164: 4616–4626.
34. Rao, N., S. Miyake, A. L. Reddi, P. Douillard, A. K. Ghosh, I. L. Dodge, P. Zhou, N. D. Fernandes, and H. Band. 2002. Negative regulation of Lck by Cbl ubiquitin ligase. *Proc. Natl. Acad. Sci. USA* 99: 3794–3799.
35. Andoniou, C. E., N. L. Lill, C. B. Thien, M. L. Lupher, Jr., S. Ota, D. D. Bowtell, R. M. Scaife, W. Y. Langdon, and H. Band. 2000. The Cbl proto-oncogene product negatively regulates the Src-family tyrosine kinase Fyn by enhancing its degradation. *Mol. Cell Biol.* 20: 851–867.
36. Miura-Shimura, Y., L. Duan, N. L. Rao, A. L. Reddi, H. Shimura, R. Rottapel, B. J. Druker, A. Tsygankov, V. Band, and H. Band. 2003. Cbl-mediated ubiquitinylation and negative regulation of Vav. *J. Biol. Chem.* 278: 38495–38504.
37. Fang, D., and Y. C. Liu. 2001. Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. *Nat. Immunol.* 2: 870–875.
38. Balagopalan, L., V. A. Barr, C. L. Sommers, M. Barda-Saad, A. Goyal, M. S. Isakowitz, and L. E. Samelson. 2007. c-Cbl-mediated regulation of LAT-nucleated signaling complexes. *Mol. Cell Biol.* 27: 8622–8636.
39. Liou, J., F. Kiefer, A. Dang, A. Hashimoto, M. H. Cobb, T. Kurosaki, and A. Weiss. 2000. HPK1 is activated by lymphocyte antigen receptors and negatively regulates AP-1. *Immunity* 12: 399–408.
40. Le Bras, S., I. Foucault, A. Foussat, C. Brignone, O. Acuto, and M. Deckert. 2004. Recruitment of the actin-binding protein HIP-55 to the immunological synapse regulates T cell receptor signaling and endocytosis. *J. Biol. Chem.* 279: 15550–15560.
41. Shui, J. W., J. S. Boomer, J. Han, J. Xu, G. A. Dement, G. Zhou, and T. H. Tan. 2007. Hematopoietic progenitor kinase 1 negatively regulates T cell receptor signaling and T cell-mediated immune responses. *Nat. Immunol.* 8: 84–91.
42. Di Bartolo, V., B. Montagne, M. Salek, B. Jungwirth, F. Carrette, J. Fourtane, N. Sol-Foulon, F. Michel, O. Schwartz, W. D. Lehmann, and O. Acuto. 2007. A novel pathway down-modulating T cell activation involves HPK-1-dependent recruitment of 14-3-3 proteins on SLP-76. *J. Exp. Med.* 204: 681–691.
43. Liu, Y. C., C. Elly, H. Yoshida, N. Bonnefoy-Berard, and A. Altman. 1996. Activation-modulated association of 14-3-3 proteins with Cbl in T cells. *J. Biol. Chem.* 271: 14591–14595.
44. Holsinger, L. J., D. M. Spencer, D. J. Austin, S. L. Schreiber, and G. R. Crabtree. 1995. Signal transduction in T lymphocytes using a conditional allele of Sos. *Proc. Natl. Acad. Sci. USA* 92: 9810–9814.
45. Ebinu, J. O., S. L. Stang, C. Teixeira, D. A. Bottorff, J. Hooton, P. M. Blumberg, M. Barry, R. C. Bleakley, H. L. Ostergaard, and J. C. Stone. 2000. RasGRP links T-cell receptor signaling to Ras. *Blood* 95: 3199–3203.
46. Roose, J. P., M. Mollenauer, V. A. Gupta, J. Stone, and A. Weiss. 2005. A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. *Mol. Cell Biol.* 25: 4426–4441.
47. Bonello, G., N. Blanchard, M. C. Montoya, E. Aguado, C. Langlet, H. T. He, S. Nunez-Cruz, M. Malissen, F. Sanchez-Madrid, D. Olive, C. Hivroz, and Y. Collette. 2004. Dynamic recruitment of the adaptor protein LAT: LAT exists in two distinct intracellular pools and controls its own recruitment. *J. Cell Sci.* 117: 1009–1016.