

# Dual Effects of Sprouty1 on TCR Signaling Depending on the Differentiation State of the T Cell<sup>1</sup>

Heonsik Choi,\* Sung-Yup Cho,<sup>†</sup> Ronald H. Schwartz,\* and Kyungho Choi<sup>2\*</sup>

Sprouty (Spry) is known to be a negative feedback inhibitor of growth factor receptor signaling through inhibition of the Ras/MAPK pathway. Several groups, however, have reported a positive role for Spry involving sequestration of the inhibitory protein c-Cbl. Thus, Spry may have various functions in the regulation of receptor-mediated signaling depending on the context. In the immune system, the function of Spry is unknown. In this study, we investigated the role of Spry1 in T cell activation. Spry1, among the four mammalian homologs, was specifically induced by TCR signaling of CD4<sup>+</sup> murine T cells. In fully differentiated Th1 clones, overexpressed Spry1 inhibited TCR signaling and decreased IL-2 production while reducing expression with specific siRNA transfection had the opposite effect, increasing IL-2 production. In contrast, in naive T cells, Spry1 overexpression enhanced TCR signaling, and increased proliferation and IL-2 production, while siRNA transfection again had the opposite effect, reducing IL-2 production following activation. The enhancing effect in naive cells was abrogated by preactivation of the T cells with Ag and APC, indicating that the history of exposure to Ag is correlated with a hierarchy of T cell responsiveness to Spry1. Furthermore, both the NF-AT and MAPK pathways were influenced by Spry1, implying a different molecular mechanism from that for growth factor receptor signaling. Thus, Spry1 uses a novel mechanism to bring about differential effects on TCR signaling through the same receptor, depending on the differentiation state of the T cell. *The Journal of Immunology*, 2006, 176: 6034–6045.

**A**ntigen recognition by the TCR initiates intracellular signal transduction by recruiting various signaling molecules to the TCR complex.

These molecules activate several sequential cascades to the nucleus, inducing transcription of new sets of genes, which are responsible for proliferation, differentiation, apoptosis, and anergy. Briefly, Src-family protein tyrosine kinases, such as Lck, are activated following TCR engagement, resulting in phosphorylation of CD3 molecules and Syk family protein tyrosine kinases (Zap70) (1, 2). Activated Zap70 phosphorylates linker for activated T cells (LAT)<sup>3</sup> and Src homology 2 (SH2)-domain containing leukocyte-specific phosphoprotein which serve an adaptor function for various molecules, including phospholipase C (PLC)- $\gamma$ 1 (3–7). Subsequently the activated PLC- $\gamma$ 1 hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol 3,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). These two

second-messenger molecules cause activation of the Ca<sup>2+</sup>-dependent pathway and the Ras/MAPK pathway, respectively, leading to nuclear translocation of transcription factors, such as NF-AT and production of new transcription factors, such as AP-1 (8, 9).

TCR engagement also triggers negative regulatory pathways for the fine tuning of TCR signaling (10–12). Protein phosphatases such as SH2 domain-containing phosphatase 1 (SHP)-1 and SHP-2 inactivate their target molecules by dephosphorylation (13, 14). Activated Src family kinases are turned off by C-terminal Src kinase (Csk), which phosphorylates their negative regulatory C-terminal tyrosine residue (15). c-Cbl, a well known E3-ubiquitin ligase, down-regulates its target (for example TCR  $\zeta$ -chain) by inducing 26S proteasome-mediated protein degradation (16). T cells also have inhibitory adaptors such as Gab2 which facilitates localization of phosphatases to the TCR complex, and Csk-binding protein, also known as phosphoprotein associated with glycosphingolipid-enriched microdomains, which cooperates with Csk (17–19). In addition to these immediate early negative regulators, some molecules are synthesized de novo following T cell activation, showing late phase feedback inhibition. The inhibitory molecules, CTLA-4 and PD-1, are such examples (18, 20).

In an effort to search for novel regulators of T cell signaling through DNA microarray screening, we found that Sprouty1 (Spry1), a known antagonist of receptor tyrosine kinase (RTK) signaling, is induced by TCR stimulation in the murine T cell clone, A.E7. Spry was initially discovered in a genetic screen as an inhibitor of *Drosophila* fibroblast growth factor (FGF) receptor signaling during trachea development (21). Subsequently, it was also found to act as an antagonist of epidermal growth factor (EGF) receptor signaling in eye and wing development and to interfere with other RTKs such as Torso and Sevenless (22, 23). These studies implied that *Drosophila* Spry might be a general inhibitor of RTK signaling and prompted investigators to pursue its mammalian homologs and their role in RTK signaling. To date,

\*Laboratory of Cellular and Molecular Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and <sup>†</sup>Department of Biochemistry and Molecular Biology/Aging and Apoptosis Research Center, Seoul National University College of Medicine, Seoul, Korea

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<sup>2</sup> Address correspondence and reprint requests to Dr. Kyungho Choi at the current address: 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

<sup>3</sup> Abbreviations used in this paper: LAT, linker for activation of T cells; SH2, Src homology 2; PLC, phospholipase C; IP<sub>3</sub>, inositol 3,4,5-triphosphate; DAG, diacylglycerol; SHP, SH2 domain-containing phosphatase 1; Csk, C-terminal Src kinase; Spry, Sprouty; RTK, receptor tyrosine kinase; FGF, fibroblast growth factor; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; FRS, fibroblast growth factor receptor substrate; PCC, pigeon cytochrome c; hSpry, human Spry; MCC, moth cytochrome c; EHAA, Eagle's, Hanks', amino acids.

four mammalian Sprys (Spry 1–4) and three Spry-related EVH1 domain proteins (sprd 1–3) have been reported (21, 24–26). Disruption of Spry2 in mouse embryonic lung, using an antisense oligonucleotide, enhanced branching morphogenesis (27). This result is similar to enhanced tracheal branching in Spry<sup>null</sup> *Drosophila*, which is due to increased FGF receptor signaling (21). Overexpression of mouse Spry2 and Spry4 inhibited FGF-mediated chicken limb bud outgrowth and angiogenesis in the mouse embryo, respectively (25, 28). In cell culture systems, overexpressed Spry1, 2, or 4 inhibited FGF, EGF, and vascular endothelial growth factor (VEGF)-mediated proliferation, differentiation and migration in various cell types including endothelial cells, HeLa cells, NIH3T3 cells, and PC12 cells (28–32). At the same time, Spry gene expression has been shown to be induced by FGF and EGF signaling in vivo and in vitro cell culture in both *Drosophila* and mammalian cells, suggesting its role in a negative feedback loop (21, 22, 25, 31). Taken together, both loss-of-function and gain-of-function studies indicate that Spry is a negative feedback regulator of RTK signaling.

Spry's inhibitory function has been largely attributed to its ability to inhibit the Ras/MAPK pathway, even though the exact site of inhibition is controversial (29, 33, 34). The most detailed mechanism of Ras/MAPK inhibition by Spry1 and 2 was reported by Hanafusa et al. (35) showing that Spry binds to the SH2 domain of Grb2 in response to FGF stimulation and thereby prevents recruitment of the Grb2-SOS complex to fibroblast growth factor receptor substrate 2 (FRS2) and SHP2. This causes inhibition of downstream events in the Ras/MAPK pathway.

Recently, however, several groups have reported that human Spry2 (hSpry2) can enhance Ras/MAPK signaling induced by EGFR using a completely different mechanism from its negative effect (36–38). Following stimulation of EGFR, c-Cbl induces ubiquitination of the receptor, targeting it for destruction by endocytosis and trafficking to lysosomes. hSpry2 abrogates this c-Cbl-mediated receptor down-regulation by sequestering c-Cbl away from the EGFR via direct binding to c-Cbl. Thus, the level of cell surface EGFR is maintained and consequently the activation of the Ras/MAPK pathway is sustained. Therefore, Sprys are not exclusively negative regulators of RTK.

The function of Spry proteins in TCR signaling has not been studied at all. After observing that Spry1 was induced by TCR signaling, we explored the role of Spry1 in the regulation of T cell activation using the TAT-fusion protein overexpression technique. Spry1 inhibited MAPK activation following TCR stimulation in murine T cell clones and was accompanied by a down-regulation of IL-2 production. At the same time, Spry1 inhibited NF-AT activation, implying a novel mechanism of action for Spry in T cells. In contrast, in naive T cells, TCR signaling, including MAPK and NF-AT activation, was enhanced by overexpression of Spry1, and this was accompanied by an increase in proliferation and IL-2 production. The enhancing effect was abolished by preactivation of naive cells with Ag and APC. These data suggest that Spry1 has differential effects on T cells depending on their activation history.

## Materials and Methods

### Mice and cell preparation

All mice were obtained from the National Institute of Allergy and Infectious Diseases contract facility at Taconic Farms, an American Association for the Accreditation of Laboratory Animal Care-accredited specific pathogen-free barrier. At the National Institutes of Health, the mice were housed in sterile caging. 5C.C7-TCR transgenic, Rag2<sup>-/-</sup> mice that specifically recognize the pigeon cytochrome *c* (PCC) peptide

81–104 and the moth cytochrome *c* (MCC) peptide 88–103, bound to I-E<sup>a</sup> or <sup>k</sup> have been described previously (39). OT-I TCR transgenic, Rag1<sup>-/-</sup> mice expressing an H-2K<sup>b</sup>-restricted receptor specific for the OVA<sub>257–264</sub> epitope (SIINFEKL) also have been described previously (40). Lymph node cells from these mice were freshly isolated and used as CD4<sup>+</sup> and CD8<sup>+</sup> naive T cells, respectively. A population of in vitro preactivated CD4<sup>+</sup> T cells was made by stimulating 5C.C7, Rag2<sup>-/-</sup> splenocytes with 1  $\mu$ M PCC peptide. After 72 h, the activated T cells were split 1 to 4, expanded in IL-2 (10 U/ml) and complete medium (45% Eagle's, Hanks', amino acids (EHAA) and 45% RPMI 1640 supplemented with 10% FCS, antibiotics, 2 mM glutamine, and 50  $\mu$ M 2-ME), and rested for at least 10 days before use.

### Cell lines

A.E7 and F1.A2 are CD4<sup>+</sup> Th1 clones specific for PCC 81–104 in the context of I-E<sup>k</sup>. They were maintained by repeated stimulation every 2–4 wk as previously described (41, 42). Briefly, cells were stimulated with whole PCC protein in the presence of irradiated (3000 rad) B10.A splenocytes as a source of APC. After 48 h, the cells were expanded in IL-2 (10 U/ml) containing complete medium and rested for at least 2 wk before use. The live cells were isolated on a Ficoll gradient and used in experiments.

The murine Th2 T cell clone D10.G4.1 (American Type Culture Collection) is CD4<sup>+</sup>, CD8<sup>-</sup>, conalbumin specific, and H-2 I-A<sup>k</sup> restricted. The cells were maintained in EHAA medium supplemented with 10% FCS, 2 mM glutamine, antibiotics, and 50  $\mu$ M 2-ME. The cells were stimulated every 10–14 days with 100  $\mu$ g/ml conalbumin (Sigma-Aldrich) plus irradiated (3000 rad) syngeneic AKR/J (The Jackson Laboratory) spleen cells as APC. Con A-stimulated rat growth factor supernatant treated with  $\alpha$ -methylmannoside (BD Biosciences) was added at a concentration of 10% to maintain the D10.G4.1 cells.

### DNA microarray

RNA was isolated from A.E7 T cell clones that were stimulated with anti-TCR for 2, 4, and 6 h or unstimulated. A reverse transcriptase reaction was performed to make cDNAs using a T7-oligo(dT) primer. Biotinylated cRNA probes were prepared from a T7 RNA polymerase-mediated in vitro transcription reaction. The probes were hybridized to Affymetrix mouse MU74A, B, and C gene chips and stained with streptavidin-PE. Hybridization and analysis of the fluorescent intensities were done at our NIAID core facility according to Affymetrix protocols (Affymetrix Microarray Suite User Guide). A microarray chip has 16 different oligonucleotides (perfect match) per gene to represent a single gene. For each oligonucleotide, a control oligonucleotide with a single base pair mismatch (mismatch) was used for the subtraction of background signal. "Average difference" represents the difference in fluorescence intensity between perfect match and mismatch (Affymetrix Microarray Suite, version 4.0).

### DNA constructs and mutagenesis

cDNA for Spry1 was amplified by RT-PCR from RNA of anti-TCR stimulated A.E7 cells and cloned into pGEM-T vector (Promega). Full-length Spry1 cDNA was reamplified via PCR and subcloned in-frame into the *KpnI* and *EcoRI* sites of the pHM6 plasmid (Roche) for mammalian expression and the pTAT-HA (a gift from Dr. S. Dowdy, University of California at San Diego, La Jolla, CA) for TAT-fusion protein production. pTAT-HA-Spry1 (Y53F) was generated by QuikChange site-directed mutagenesis (Stratagene). All the sequences were confirmed by automated DNA sequencing.

### Expression and Purification of TAT-fusion proteins

The expression and purification of recombinant TAT-fusion proteins was performed as described (43). Briefly, the recombinant TAT-Spry1 proteins were expressed in the BL21 (DE3) pLysS strain of *Escherichia coli* by 18 h culture at 37°C. Bacterial cell pellets were washed with PBS and sonicated in lysis buffer (6 M guanidine hydrochloride, 20 mM HEPES (pH 8.0), 10 mM imidazole, 100 mM NaCl). The cell lysate was centrifuged and the supernatant was loaded on a nickel-nitrilotriacetic acid column (Qiagen) and washed successively with lysis buffer, binding buffer (20 mM HEPES, 10 mM imidazole, 100 mM NaCl, 8 M urea) and washing buffer (20 mM HEPES, 20 mM imidazole, 100 mM NaCl, 8 M urea). Recombinant proteins were eluted with elution buffer (20 mM HEPES, 330 mM imidazole, 100 mM NaCl, 8 M urea) and desalted on a PD-10 column (Amersham Biosciences) into RPMI 1640 medium containing 10% glycerol. The Y53F mutant of TAT-Spry1 (Tyr<sup>53</sup>→Phe) was also expressed and purified using the same method. TAT-GFP expressing bacteria were a gift from Dr. Dowdy. After overnight bacterial culture, TAT-GFP protein expression

was induced with 0.4 mM IPTG and the bacterial cell pellets were sonicated in 8 M urea-containing binding buffer. The purification procedure was similar to that for TAT-Spry1. The final TAT-protein concentration was measured by SDS-PAGE in comparison with BSA standards and Coomassie blue staining. Yields were typically around 200  $\mu$ g from 1L bacterial cell culture for TAT-Spry1 and 22 mg from 1L culture for TAT-GFP.

#### Labeling of the recombinant protein

The recombinant protein was labeled with Alexa Fluor 488 dye (Molecular Probes) according to the manufacturer's instructions. Cells were incubated with labeled protein for 2 h and washed with ice-cold FACS buffer (PBS supplemented with 0.2% BSA and 0.1%  $\text{NaN}_3$ ) three times and then analyzed by flow cytometry on a BD FACSCalibur (BD Biosciences). For the confocal microscopy study, cells were incubated under the same conditions. After incubation, cells were washed three times with ice-cold PBS and analyzed. Images were collected on a Leica TCS-NT/SP1 confocal microscope (Leica Microsystems) using a  $\times 63$  oil immersion objective NA 1.32. Fluorochromes were excited using an argon laser at 488 nm for FITC. Differential interference contrast images were collected simultaneously with the fluorescence images using the transmitted light detector. Images were processed using the Leica TCS-NT/SP software (version 2.61.1347).

#### Proliferation and cytokine assays

A total of  $2 \times 10^4$  cells were cultured in triplicate in complete EHAA/RPMI 1640 medium supplemented with 10% FCS in 96-well plates coated with anti-CD3 mAb (145-2C11; BD Pharmingen) plus soluble anti-CD28 mAb (37-51) ascites at a dilution of 1/2500. After 48 h, one-half of the culture supernatant was collected to measure cytokines and the remaining cells and medium were pulsed with 1  $\mu$ Ci of [ $^3\text{H}$ ]thymidine per well for an additional 24 h. Cells were harvested using a Brandel 96-well harvester and the radioactivity was counted in a Wallac Trilux 1450 scintillation counter. Secreted cytokines were measured using ELISA kits (R&D Systems) according to the manufacturer's instructions.

#### CFSE dye dilution assay

For analysis of cell division, lymph node T cells from 5C.C7, Rag2 $^{-/-}$  mice were isolated and labeled with CFSE (Molecular Probes) at a final concentration of 1  $\mu$ M in PBS for 15 min at 37°C. The labeled cells were activated for 60 h by plate-bound anti-CD3 (1 or 10  $\mu$ g/ml) plus anti-CD28. Dilution of CFSE was measured by flow cytometry and mean division number was quantitated as described previously (44).

#### Western blot analysis

The protein concentrations of samples were determined using a BCA protein assay kit (Pierce). The same amounts of protein were separated on SDS-PAGE and transferred to nitrocellulose membranes. Specific proteins were detected using the following primary Abs: anti-Spry2 (Upstate Biotechnology), anti-Spry3 (Upstate Biotechnology), anti-Spry4 (Santa Cruz Biotechnology), anti-phospho-Erk (Santa Cruz Biotechnology), anti-NF-AT c2 (Santa Cruz Biotechnology), anti-Lamin B (Santa Cruz Biotechnology), and anti-Spry1. Anti-Spry1 was either purchased from Zymed Laboratories or generated by immunizing rabbits with the N-terminal peptide: AVEGRQLDYDRDTQ (aa 20–34 of Spry1; Bethyl Laboratories). Primary Abs were detected by anti-mouse or anti-rabbit Abs coupled with HRP (both from Amersham Biosciences) or anti-goat Ab coupled with HRP (Santa Cruz Biotechnology) followed by a chemiluminescence reaction (Supersignal West Pico; Pierce). Blots were stripped and reprobed with anti-actin (Sigma-Aldrich) or anti-MAPK 1/2 (Upstate Biotechnology) to monitor equal loading.

#### Nuclear fractionation

Ten million lymph node T cells from 5C.C7, Rag2 $^{-/-}$  mice were stimulated with plate-bound anti-CD3 mAb plus anti-CD28 mAb for 24 h. Cells were harvested, washed twice in cold PBS, and suspended in 200  $\mu$ l of buffer A (10 mM KCl, 10 mM HEPES (pH 8.0), 0.1 mM EGTA (pH 8.0), 0.1 mM EDTA (pH 8.0), protease inhibitor mixture (Roche), 1 mM DTT, 1 mM sodium orthovanadate, and 0.5% Nonidet P-40) for 5 min on ice. After immediate centrifugation at 12,000 rpm, the pellet was washed twice in buffer A and incubated with 50  $\mu$ l of buffer C (420 mM NaCl, 20 mM HEPES (pH 8.0), 1 mM EGTA (pH 8.0), 1 mM EDTA (pH 8.0), protease inhibitor mixture, 1 mM DTT, and 1 mM sodium orthovanadate) for 2 h with frequent vortexing on ice. The nuclear fraction was collected by centrifugation at 12,000 rpm for 15 min.

#### siRNAs and nucleofection

Five million lymph node T cells were resuspended in 100  $\mu$ l of Nucleofector Solution (Mouse T Cell Nucleofector kit: VPA-1006; Amaxa). Two micrograms of siRNA was added and mixed well. The cell-siRNA mixture was transferred to an electroporation cuvette and placed in the Nucleofector II device (Amaxa). Nucleofection of the cells was accomplished using the U-14 program. Cells were then transferred to 500  $\mu$ l of prewarmed medium (RPMI 1640, 10% FCS, 2 mM glutamine, and 50  $\mu$ M 2-ME) and incubated for 10 min in a 37°C water bath. Samples were transferred to 24-well plates containing 1 ml of prewarmed medium. The cells were incubated for 4 h in a 37°C incubator containing 5%  $\text{CO}_2$  and then harvested and counted. To investigate the induction of protein, cells were stimulated with plate-bound Ab in 6 well plates for 16 h. For cytokine assays, an aliquot of the same cells was stimulated with either peptide-pulsed APCs or plate-bound Ab in 96-well plates for 48 h. The siRNA sequences specific for mouse Spry1 (AAGCAGCUGCUGGUGGAAGAC) and a nontargeting siRNA (UAGCGACUAAACACAUCAA) were synthesized and annealed by the manufacturer (Dharmacon).

## Results

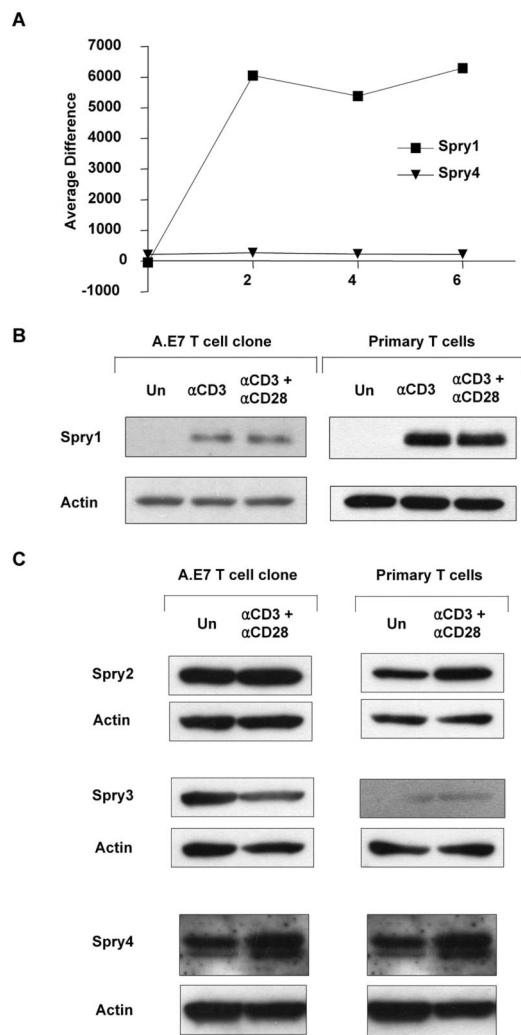
#### Spry1 is induced by TCR stimulation

The A.E7 Th1 clone was stimulated with plate-bound anti-TCR Ab to screen for TCR signal-induced genes. Isolated mRNA was converted into biotinylated cRNA probes and then hybridized to an oligonucleotide microarray from Affymetrix. The level of Spry1 mRNA was increased as early as 2 h after stimulation and maintained thereafter (Fig. 1A). In contrast, the mRNA for Spry4 was constitutively expressed at a low level throughout the entire 6 h time period. Induction of Spry1 protein was also confirmed in a Western blot (Fig. 1B). No Spry1 protein was present in the resting state, but a considerable amount of protein was detected upon stimulation with anti-CD3 overnight. To determine whether costimulation affected the amount of Spry1 induced, anti-CD28 was added along with anti-CD3 mAb. No additive or negative effects were observed compared with anti-CD3 treatment alone (Fig. 1B). This same pattern of induction was observed in primary naive T cells from 5C.C7-TCR transgenic mice (Fig. 1B). We also checked the protein expression of other members of the Spry family by Western blotting. In contrast to Spry1, Spry2, 3, and 4 were all constitutively expressed in the resting state in A.E7 T cells and not induced by stimulation with anti-CD3 and anti-CD28 after 16 h. Primary naive T cells showed a similar pattern with only a slight increase in Spry2 and 4 after stimulation. Spry3 expression was barely detectable compared with what was observed in A.E7 T cells (Fig. 1C). Therefore, we conclude that Spry1 is the only Spry homolog that is strongly induced by TCR engagement and that this expression is not augmented by anti-CD28 signaling.

#### TAT-Spry1 inhibits IL-2 production in T cell clones

To explore the effect of Spry1 induction on T cell activation, we overexpressed Spry1 in unstimulated A.E7 T cells. Conventional plasmid transfection methods using electroporation or liposome-based reagents give low transfection efficiencies with unstimulated T cell clones and primary T cells. To overcome this problem we used the TAT-fusion protein transduction technique that has been shown to work with virtually all cells, including murine splenocytes (45–48). An 11-aa sequence derived from the HIV TAT protein served as the protein transduction domain, which leads the tethered polypeptide into the cell in a receptor-independent manner. TAT-hSpry2 has previously been efficiently transduced into human epithelial cells using this method and showed a functional phenotype (32). First, we checked to see whether TAT-fusion proteins could be transduced into murine T cells. Recombinant TAT-Spry1 protein was purified and added to resting A.E7 cells at various

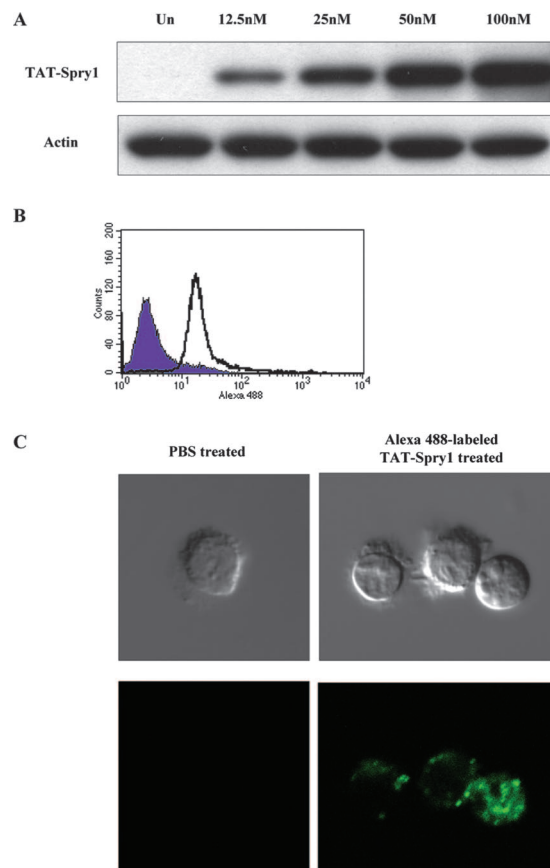




**FIGURE 1.** mSpry1 is specifically induced by TCR stimulation among Spry homologs. *A*, Resting A.E7 cells were stimulated with plate-bound anti-TCR Ab for 2, 4, or 6 h. Biotinylated cRNA probes were generated from isolated total RNA and hybridized to microarray chips. Amounts of Spry1 and 4 mRNA were evaluated and are represented as the fluorescent intensity (average difference). *B* and *C*, Resting A.E7 T cells or freshly isolated LN cells from 5C.C7-TCR transgenic, Rag2<sup>-/-</sup> mice were stimulated with anti-CD3 (10  $\mu$ g/ml) or anti-CD3 plus anti-CD28 for 16 h. The cell lysates were electrophoresed and transferred to a nitrocellulose membrane. The blots were probed with anti-Spry1 (*B*), 2, 3, or 4 (*C*). To prove equal loading, the same blots were stripped and reprobed with anti-actin.

concentrations. After 2 h of incubation, the cells were washed with ice-cold PBS and the amount of Spry1 measured in a Western blot using anti-Spry1 Ab. Transduced Spry1 protein was detected and the transduction was dose dependent (Fig. 2*A*). The transduction efficiency was over 90% when the cells were treated with fluorescent dye-labeled TAT-Spry1 and analyzed by flow cytometry (Fig. 2*B*). The presence of TAT proteins inside the cell was further confirmed using confocal microscopy (Fig. 2*C*) and z-axis analysis (data not shown). Therefore, TAT-Spry1 can be efficiently transduced into murine T cells.

Next, we examined the effect of TAT-Spry1 on A.E7 cytokine production and proliferation induced by TCR stimulation. Spry1 significantly blocked IL-2 production (Fig. 3*A*). The maximum amount of IL-2 secreted was decreased ~65%, while a negative control protein, TAT-GFP, had minimal effect. Sim-

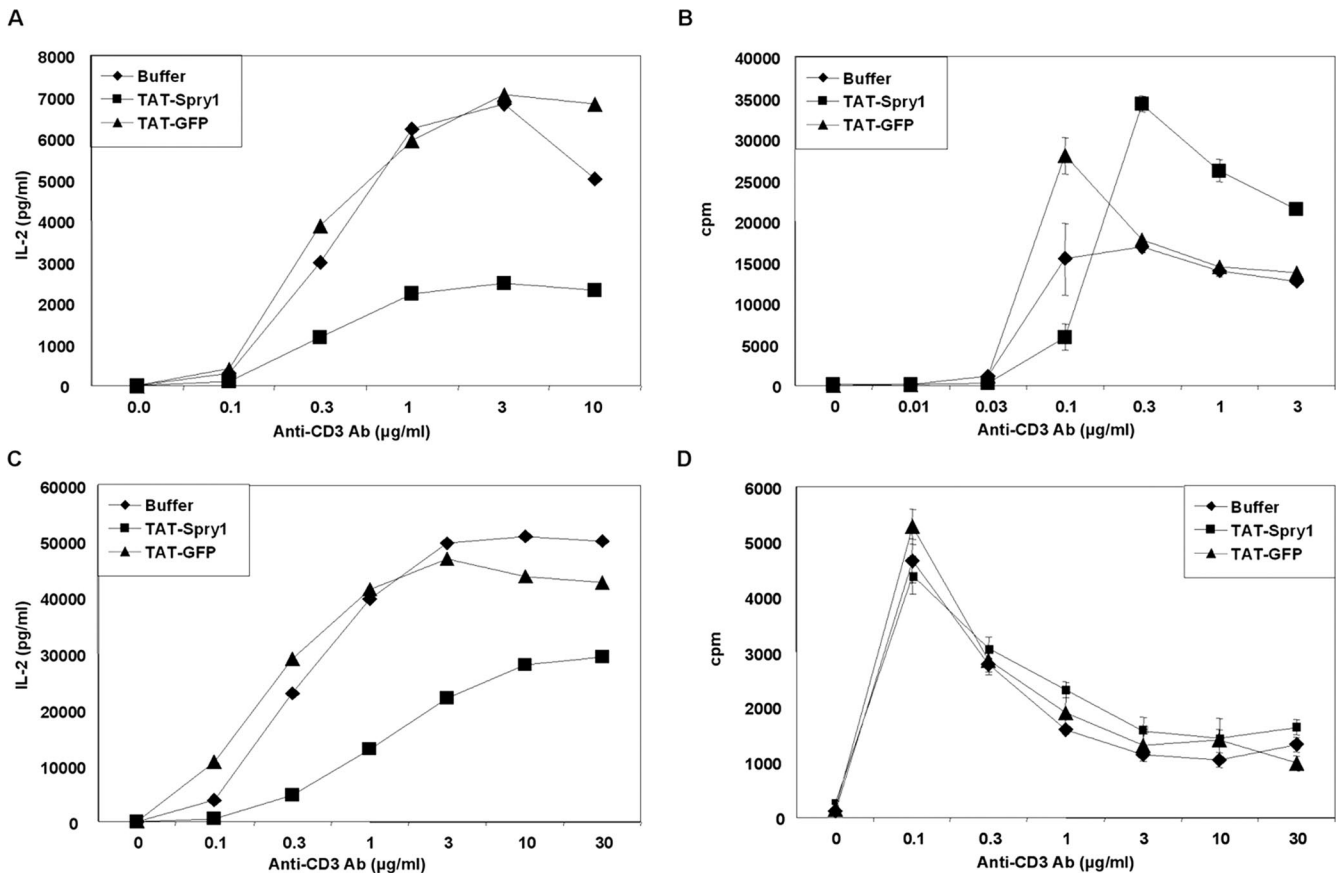


**FIGURE 2.** A.E7 cells are efficiently transduced with TAT-Spry1 proteins. *A*, After A.E7 cells were incubated with various concentrations of TAT-Spry1 protein for 2 h at 37°C, the cells were washed with PBS and subjected to Western blotting with anti-Spry1 or anti-actin. *B* and *C*, A.E7 cells were incubated with Alexa Fluor 488-labeled TAT-Spry1 protein for 2 h at 37°C. After washing, the efficiency of protein transduction was analyzed by flow cytometry (*B*) or confocal microscopy (*C*). In *B*, the filled region represents the PBS-treated population and the open region represents the Alexa 488-labeled TAT-Spry1-treated population. In *C*, the upper panels show the differential interference contrast images; the lower panels show the cells illuminated at 488 nm for FITC.

ilar results were obtained with a different Th1 clone, F1.A2 (Fig. 3*C*), indicating that these results are not limited to a single clone. In the case of proliferation, TAT-Spry1 treatment showed modest inhibition in A.E7 cells and no effect in F1.A2 T cells (Fig. 3, *B* and *D*). Production of other cytokines, such as IFN- $\gamma$ , MIP-1 $\alpha$ , and IL-3 in A.E7 T cells and IL-4 in D10.G4.1 cells (a conalbumin-specific Th2 T cell clone), were not affected by TAT-Spry1 (data not shown), indicating that IL-2 is the most sensitive readout of the effect of Spry1 on T cell clones. Therefore, Spry1 is induced following TCR stimulation and overexpressed Spry1 inhibits IL-2 production in Th1 clones. These findings are consistent with previous reports in other cell types which showed that the Spry family of proteins can act as a negative feedback regulator of RTKs.

#### Overexpression of Spry1 inhibits both MAPK and NF-AT activation following TCR stimulation

The TCR delivers its signal to the nucleus by various biochemical pathways. One of the potential target pathways for Spry1 inhibition is Ras-mediated MAPK activation, because Spry proteins have been shown to inhibit this pathway downstream of



**FIGURE 3.** Overexpressed Spry1 inhibits IL-2 production in CD4<sup>+</sup> Th1 clones. *A–D*, A.E7 cells (*A* and *B*) or F1.A2 cells (*C* and *D*) were incubated with TAT proteins (100 nM) or buffer alone for 2 h at 37°C and then stimulated with various concentrations of plate-bound anti-CD3 plus anti-CD28 for 72 h with [<sup>3</sup>H]thymidine pulsing for the last 24 h. The amount of secreted IL-2 was measured from an aliquot of 48 h culture supernatant by ELISA (*A* and *C*). Cells were harvested at the end of cultures to measure incorporated radioactivity (*B* and *D*).

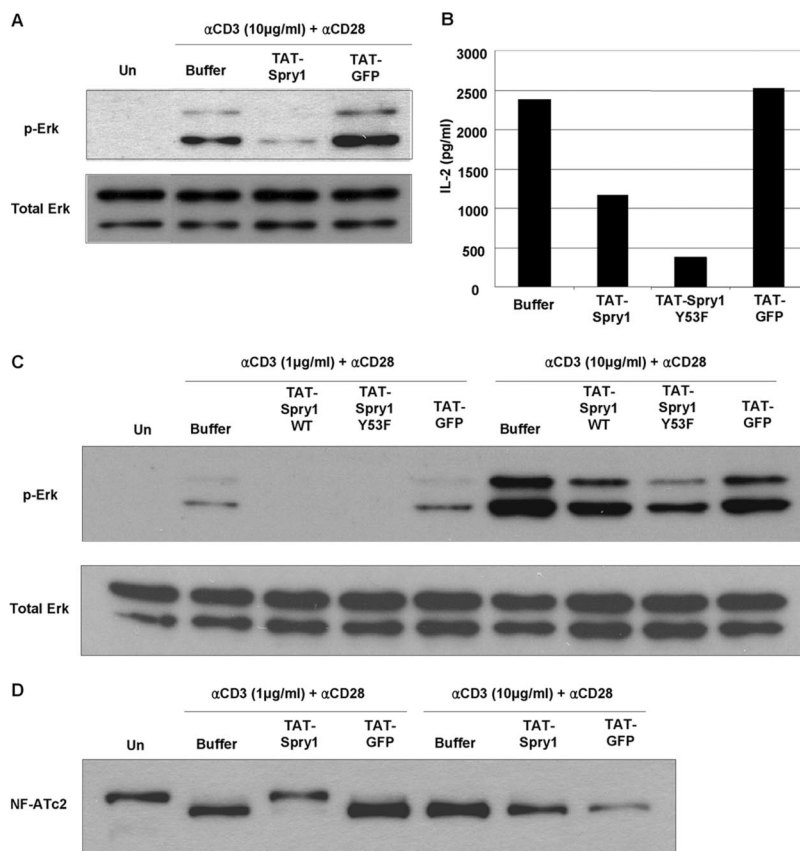
RTKs at various levels. We tested the effect of TAT-Spry1 on MAPK phosphorylation using an anti-phospho-Erk immunoblot. TAT-Spry1 reduced MAPK phosphorylation following TCR stimulation as expected (Fig. 4*A*). TAT-GFP had no effect. The most well-defined mechanism of inhibition of the Ras/MAPK pathway by Spry1 is thought to be phosphorylation at a conserved tyrosine (Tyr<sup>53</sup>) in Spry1 following FGF stimulation. Phosphorylated Spry1 then binds to the SH2 domain of Grb2, sequestering it and SOS from the FRS-SHP2 complex and thus turning off downstream Ras activation. A mutant protein that cannot be phosphorylated on its conserved Tyr does not bind Grb2 and acts as a dominant negative, inducing prolonged activation of ERK in response to FGF. To see whether this mechanism is responsible for the inhibition of the MAPK activation following TCR signaling, we mutated tyrosine 53 of Spry1 to a phenylalanine and purified the TAT-Y53F Spry1 protein. Interestingly, the mutant protein still showed an inhibitory effect, similar to that of the wild-type TAT-Spry1, in both IL-2 production and MAPK activation (Fig. 4, *B* and *C*). In fact, the mutant showed a slightly more profound inhibition in both cases. These results demonstrate that phosphorylation of Tyr<sup>53</sup> is not responsible for the inhibition of TCR-induced MAPK activation and IL-2 production, implying a different mechanism.

A second important pathway for IL-2 transcription in T cells is the Ca<sup>2+</sup>-dependent activation of NF-AT. TCR engagement triggers Ca<sup>2+</sup> flux into the cytosol and cytosolic NF-AT is then dephosphorylated by activated calcineurin, leading to translo-

cation of NF-AT into the nucleus. We examined the effect of TAT-Spry1 on dephosphorylation of NF-AT by TCR signaling. Surprisingly, TAT-Spry1 inhibited NF-AT dephosphorylation at a low dose of anti-CD3 (Fig. 4*D*). Inhibition of nuclear translocation of NF-AT was also observed using Western blotting of nuclear fractions (data not shown). However, this inhibitory effect was not seen at a higher dose (10 μg/ml). Because the MAPK activity was strongly affected even at this high dose of anti-CD3, the effect of Spry1 on NF-AT inhibition appears to be weaker than that of its MAPK inhibition. Nonetheless, these results are consistent with the conclusion that Spry1 does not work solely at the level of Grb2.

#### *Spry1 enhances TCR-induced proliferation and cytokine production in naive T cells*

One of the advantages of the TAT-fusion protein technique is that the transduction process is quick and affects most of the cells. This makes it suitable for transduction of naive T cells, which have a short life-span in *in vitro* cultures and normally a low transfection efficiency (48). To see whether overexpression of Spry1 could also affect naive T cells, we isolated lymph node T cells from 5C.C7 TCR transgenic mice on a Rag2<sup>-/-</sup> background. Flow cytometry analysis showed that 94% of the cells were CD44<sup>low</sup> and CD62L<sup>high</sup> CD4<sup>+</sup> T cells. Spry1 was shown earlier to be induced in this population (Fig. 1*B*) and TAT-Spry1 could be efficiently transduced (data not shown).



**FIGURE 4.** Spry1 inhibits TCR-mediated MAPK and NFAT activation. *A* and *D*, After A.E7 cells were transduced with TAT proteins (100 nM) or buffer alone for 2 h, cells were stimulated with plate-bound anti-CD3 plus anti-CD28 for 15 min (*A*) or 3 h (*D*). Western blotting was performed and the membranes were probed with anti-phospho-Erk (*A*) or anti-NFATc2 (*D*), respectively. *B* and *C*, A.E7 cells transduced with TAT-GFP (100 nM), TAT-Y53F-Spry1 (100 nM), the wild-type TAT-Spry1 (100 nM) or buffer alone, were stimulated with plate-bound anti-CD3 (10  $\mu$ g/ml) plus anti-CD28 for 2 days to measure secreted IL-2 (*B*) or 15 min to measure Erk phosphorylation by Western blotting (*C*).

To explore the role of overexpressed Spry1 in naive T cells, TAT-Spry1 proteins were incubated with naive T cells for 2 h and then stimulated with various concentrations of plate-bound anti-CD3 mAb and a constant concentration of anti-CD28 mAb. Surprisingly, TAT-Spry1 treated cells showed enhanced [ $^3$ H]thymidine incorporation and IL-2 production (Fig. 5, *A* and *B*). The proliferative dose-response curve was shifted at least 10-fold toward lower concentrations. To confirm that the enhanced [ $^3$ H]thymidine incorporation was the result of enhanced proliferation, a CFSE-dilution assay was also performed. Cells were labeled with CFSE and then stimulated for 60 h. CFSE dilution profiles were analyzed by flow cytometry. In control groups 50% of the cells had not yet divided at all at this time point, whereas the majority of TAT-Spry1 overexpressed cells had divided at least once (Fig. 5*C*, *left panel*). The calculated mean division number of TAT-Spry1-treated cells was >3-fold higher than control cells at a low dose of anti-CD3 stimulation (Fig. 5*D*). There was no difference in the percentage of annexin V-positive cells between the two groups (data not shown). This means that the increase in [ $^3$ H]thymidine incorporation represents enhanced proliferation, rather than enhanced cell survival. To determine whether this enhancing effect was due to the differences in the amount of recombinant protein transduced, we tried various concentrations of recombinant protein. The enhancing effect of the protein was dose dependent, but inhibition (the effect observed in the A.E7 T cell clone) was not observed at any concentration tested (data not shown).

To see whether this phenomenon was restricted to CD4 T cells, we isolated naive CD8 $^+$  T cells from lymph nodes of OT-I OVA-specific TCR transgenic mice on a Rag1 $^{-/-}$  background and performed the same experiment. TAT-Spry1 treated OT-I T cells showed a similar enhancement of proliferation (Fig. 5*E*). TAT-Spry1-treated OT-I naive T cells also produced

much higher amounts of IFN- $\gamma$  (Fig. 5*F*), showing that the enhancement of cytokine production is not limited to CD4 $^+$  helper T cells.

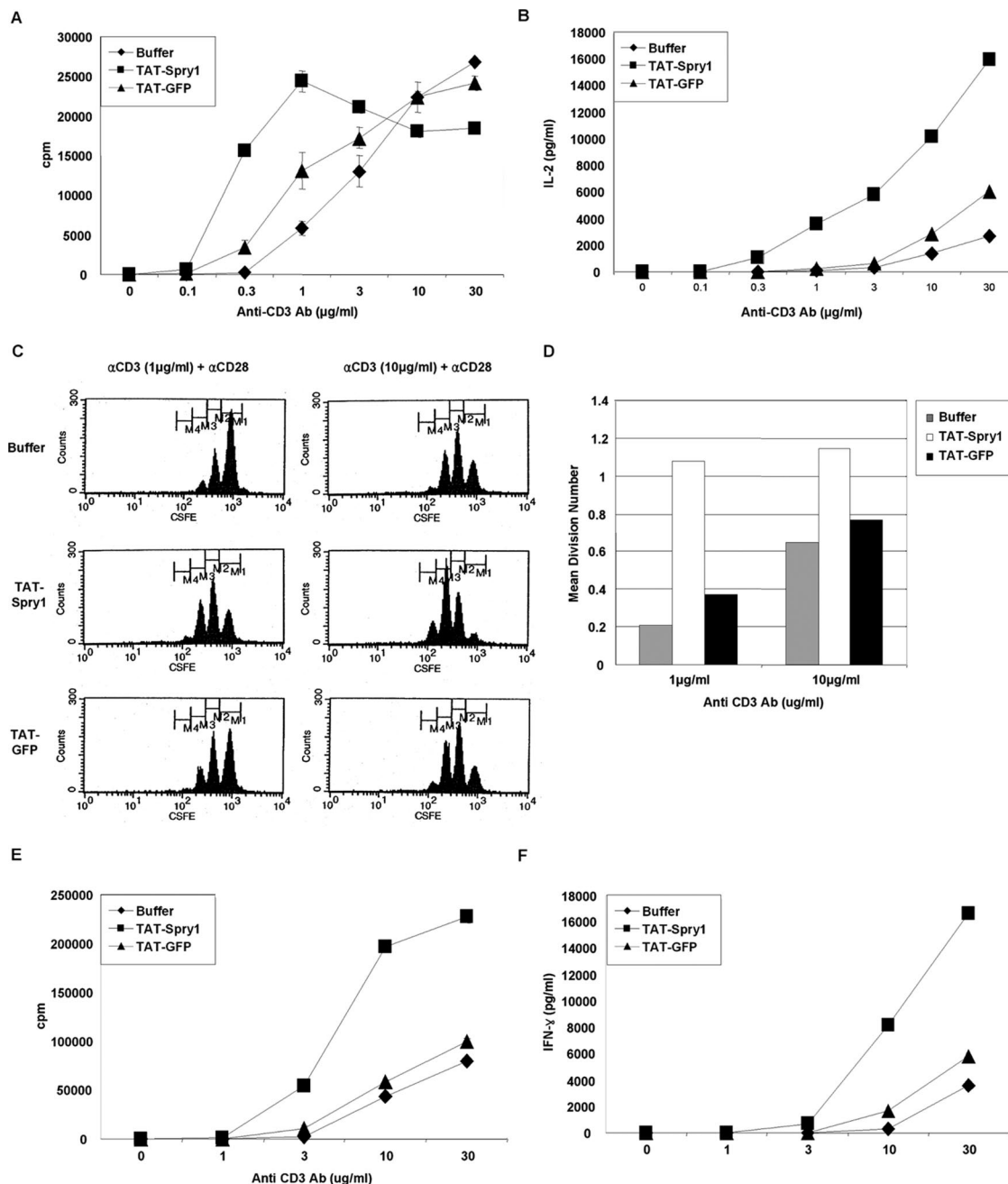
#### *Spry1 enhances TCR-mediated MAPK and NF-AT activation in naive T cells.*

To determine the site of action of Spry1 in naive T cells, we performed the same biochemical studies as described earlier. We stimulated TAT-Spry1-treated naive T cells with a 10  $\mu$ g/ml dose of plate-bound anti-CD3 mAb and anti-CD28 mAb for various times and phosphorylated MAPK was detected in a Western blot. The results show a modest enhancement of phosphorylation of MAPK in Spry1-overexpressed cells (Fig. 6*A*). We also isolated the nuclear fraction of stimulated cells and determined the amount of translocated NF-AT by Western blotting. Spry1 also enhanced nuclear translocation of NF-AT (Fig. 6*B*). These results suggest that Spry1 works at an early proximal level of TCR signaling before the bifurcation into the NF-AT and Ras pathways.

We also checked whether the Y53F Spry1 mutant showed a different effect from that of the wild-type protein. This experiment revealed the same enhancing effect as the wild type, which implies that the phosphorylation of tyrosine 53 does not play a role in the enhancement of TCR signaling (Fig. 6*C*).

#### *Spry1-specific siRNA suppresses the induction of protein and produces the opposite functional effects*

To confirm the functional roles of Spry1, we next inhibited the expression of Spry1 in A.E7 T cell clones and CD4 $^+$  naive T cells by RNA interference. Specific murine Spry1 siRNA or nontargeting control siRNA was delivered to T cells by nucleofection. Transfection of the cells with specific siRNA reduced the level of Spry1 by >40%, but it did not change the level of the control



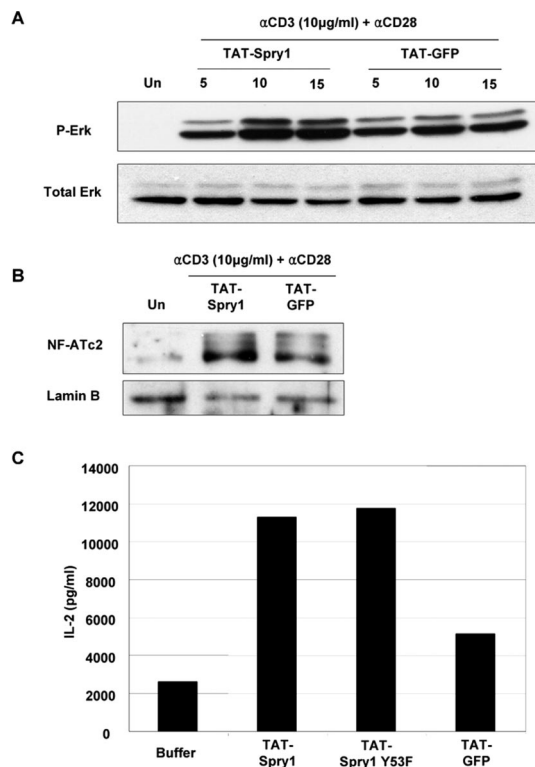
**FIGURE 5.** Overexpressed Spry1 enhances proliferation and cytokine production in naive T cells. *A, B, E, and F*, CD4<sup>+</sup> naive T cells (*A and B*) from 5C.C7-TCR transgenic, Rag2<sup>-/-</sup> mice or CD8<sup>+</sup> naive T cells (*E and F*) from OT-1 TCR-transgenic, Rag1<sup>-/-</sup> mice were transduced with TAT-proteins (100 nM) or buffer alone for 2 h and then stimulated with various concentrations of plate-bound anti-CD3 plus anti-CD28 for 72 h with [<sup>3</sup>H]thymidine pulsing for the last 24 h. The amount of secreted IL-2 (*B*) or IFN-γ (*F*) was measured from an aliquot of 48-h culture supernatant by ELISA. Cells were harvested at the end of culture to measure incorporated radioactivity (*A and E*). *C and D*, CFSE-labeled CD4<sup>+</sup> naive T cells were transduced with TAT proteins (100 nM) or buffer alone and then stimulated with plate-bound anti-CD3 (10 μg/ml) plus anti-CD28 for 60 h. Cell division was evaluated by flow cytometry (*C*) and quantified as mean division number (*D*).

protein actin (Fig. 7A). Inhibition of Spry1 expression increased the release of IL-2 from anti-CD3 stimulated A.E7 T cells by 2-fold (Fig. 7B). In contrast, the same siRNA inhibited IL-2 production by anti-CD3-stimulated CD4<sup>+</sup> naive T cells (Fig. 7C). Identical results were observed when cells were activated with their cognate peptide (MCC)-pulsed APCs (Fig. 7D). Thus, the dual effect of Spry1 in two different T cell types was recapitulated in a loss-of-function study, showing that Spry1 plays a significant role in TCR signaling.

#### *Preactivation of naive T cells abrogates the enhancing effect of TAT-Spry1*

After they finish their development in the thymus, naive T cells circulate in the periphery in a G<sub>0</sub> state until they encounter Ag. In contrast, Th1 clones are fully differentiated T effector/memory cells specializing in the production of certain cytokines. The conversion of naive T cells into Th1 clones requires multiple exposures to Ag in vitro, resulting in extensive proliferation and



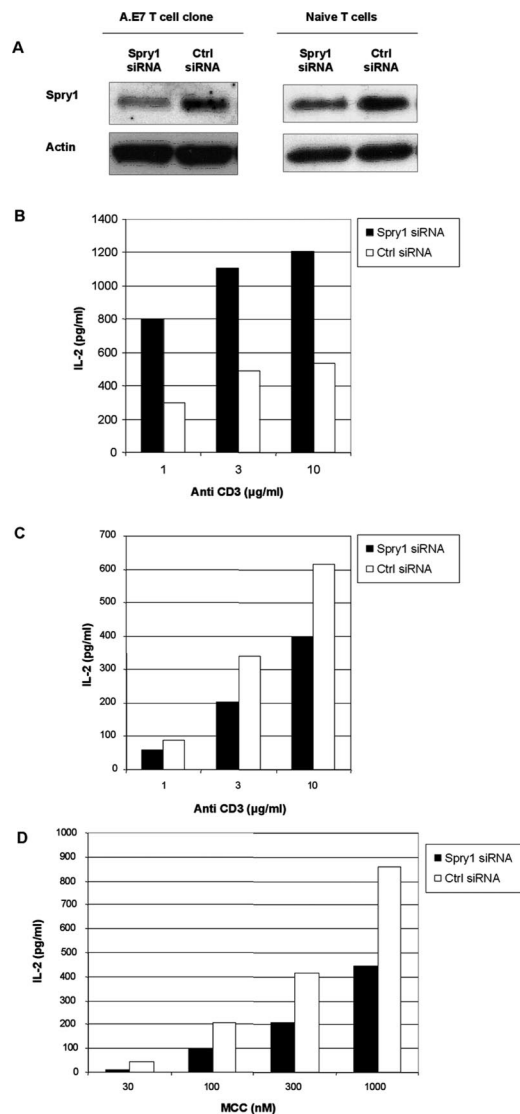


**FIGURE 6.** Spry1 enhances TCR-mediated MAPK and NFAT activation in naive T cells. *A*, CD4<sup>+</sup> naive T cells transduced with TAT-proteins (100 nM) were stimulated with anti-CD3 (10 μg/ml) plus anti-CD28 for the indicated time and cell lysates were subjected to Western blotting with anti-phospho-Erk. *B*, The same experiment was performed as in *A* except that cells were stimulated for 24 h. Nuclear fractions were isolated and the same amounts of protein were electrophoresed and blotted onto a nitrocellulose membrane. The membrane was probed with anti-NFATc2 and anti-Lamin B as a loading control. *C*, CD4<sup>+</sup> naive T cells transduced with TAT-Y53F-Spry1 (100 nM) or wild-type TAT-Spry1 (100 nM) were stimulated with anti-CD3 (10 μg/ml) plus anti-CD28 for 48 h and the amounts of secreted IL-2 were measured by ELISA.

differentiation to cytokine production. We suspected that the contrasting effect of TAT-Spry1 on naive T cells and Th1 clones might stem from this difference in Ag activation and differentiation. If that were the case, we predicted that the activation of naive T cells with Ag and APC might reverse the enhancing effect of TAT-Spry1 seen in naive T cells. To test this possibility, we stimulated 5C.C7 TCR-transgenic naive T cells with PCC and APC in vitro for 3 days and then rested the activated T cells for 10 days without Ag. This resting, preactivated T cell population was then incubated with TAT-Spry1 and proliferation and IL-2 production determined in response to anti-CD3 mAb plus anti-CD28 mAb. As expected, preactivation abrogated the enhanced proliferation and increased IL-2 production seen with TAT-Spry1-treated naive T cells (Fig. 8). Moreover, subtle negative effects of TAT-Spry1 were observed for both IL-2 production and proliferation, compared with the TAT-GFP control. These data suggest that T cell activation by Ag and APC, and the subsequent differentiation process, change the effect of overexpressed Spry1 on T cell responsiveness from a positive one to a negative one. Thus, the preactivated T cell population may represent an intermediate step in this differentiation process.

## Discussion

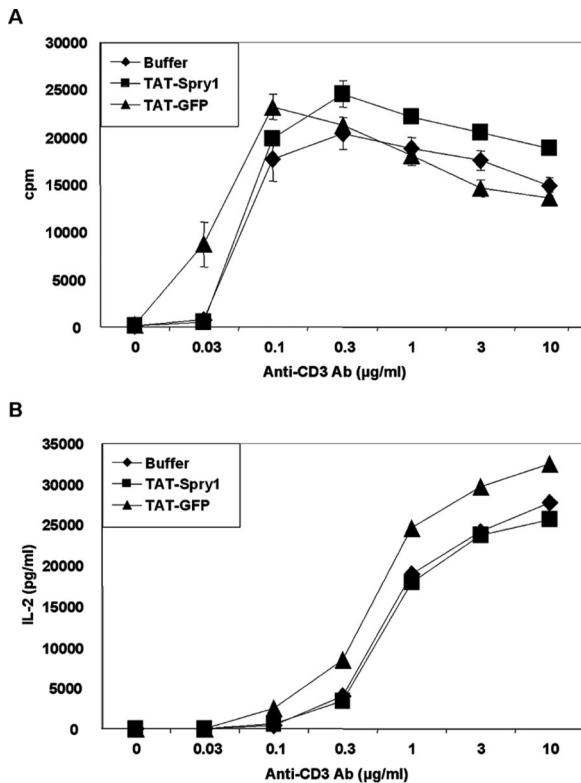
Much attention has been paid to Spry since it was first identified in *Drosophila*. Most of the studies, from *Drosophila* to mammalian



**FIGURE 7.** Spry1-specific siRNA suppresses the induction of Spry1. *A*, The A.E7 T cell clone or CD4<sup>+</sup> naive T cells from 5C.C7-TCR transgenic, Rag2<sup>-/-</sup> mice were transfected with either Spry1-specific siRNA or non-targeting control siRNA. Cells were stimulated with plate-bound anti-CD3 plus anti-CD28 for 16 h and cell lysates were electrophoresed and transferred to a membrane. The blots were probed with anti-Spry1. To prove equal loading, the same blots were stripped and reprobed with anti-actin. After transfection, A.E7 cells (*B*) or CD4<sup>+</sup> naive T cells (*C*) were stimulated with various concentrations of anti-CD3 Ab plus anti-CD28 for 48 h and IL-2 ELISAs performed on the supernatants. Alternatively, CD4<sup>+</sup> naive T cells were stimulated with MCC peptide-pulsed APCs (*D*).

cells, have characterized this protein as a negative regulator of RTK signaling, predominantly affecting the Ras/MAPK pathway. However, recently, several papers have shown that it may also act positively on RTK signaling depending on the context, raising the possibility that Spry can act at multiple sites in different cell types. In this report, we investigated the role of Spry1 in TCR signaling using both TAT overexpression and siRNA knockdown strategies. In fully differentiated Th1 T cell clones, Spry1 overexpression had a negative effect on TCR signal-mediated MAPK activation and IL-2 production while siRNA inhibition enhanced IL-2 production. In contrast, in undifferentiated naive T cells, overexpression enhanced TCR signaling, IL-2 production and proliferation, while siRNA inhibited IL-2 production, mimicking the bipolar effects





**FIGURE 8.** Enhancing effect of Spry1 is abrogated in preactivated T cells. *A*, Preactivated T cells were generated from splenocytes of 5C.C7-TCR transgenic, Rag2<sup>-/-</sup> mice which were activated with Ag for 3 days, followed by 14 days of rest. This population of cells was transduced with TAT proteins (100 μM) or buffer alone for 2 h. Cells were stimulated with various concentrations of plate-bound anti-CD3 plus anti-CD28 for 72 h with [<sup>3</sup>H]thymidine pulsing for the last 24 h. Cells were harvested at the end of culture to measure incorporated radioactivity. *B*, The amount of secreted IL-2 was measured by ELISA in an aliquot of 48-h culture supernatant from *A*.

that have been shown for EGF-induced MAPK activation in epithelial cell lines. Moreover, this enhancing effect was abrogated in preactivated T cells following a single exposure to Ag, implying that the responsiveness of T cells to Spry1 can change as T cells experience Ag and differentiate.

We initially noticed that Spry1 mRNA was induced by TCR signaling in a DNA microarray analysis and speculated that Spry1 could act as a negative feedback inhibitor for TCR signaling. The induction of Spry1 at both the mRNA and protein levels was isoform-specific up to 16 h after stimulation. Interestingly, in resting T cells, we did not detect any Spry1 protein, whereas the Spry2, 3, and 4 proteins were constitutively expressed. These findings indicate that the expression of only Spry1 is tightly regulated by TCR signaling. Furthermore, even though Spry1 mRNA induction peaked at 2 h of stimulation, we could not detect proteins until after overnight stimulation (data not shown), suggesting that the expression of Spry1 may be controlled at the translational (or posttranslational) level as well as at the transcriptional level. Because Spry1 seemed to be specifically regulated during T cell activation, we were prompted to investigate the function of this protein.

Sprys have been generally described to perform an inhibitory role for RTK signaling, acting on the Ras/MAPK pathway. However, the more work on Sprys that is done, the more complexities in the mode of action they reveal. First, even though Sprys target the MAPK pathway, the site of action is different for various

growth factors. Two of the best-defined examples are from FGF receptor and VEGF receptor signaling. In the former signaling, Spry1 and 2 are phosphorylated at Tyr<sup>53</sup> and Tyr<sup>55</sup>, respectively, after receptor engagement. Subsequently, phosphorylated Spry1 and 2 bind to the Grb2-SOS complex and sequester it from the upstream FRS2-SHP2 complex, thereby stopping downstream signaling (35). Phosphorylation of Tyr<sup>53/55</sup> is essential for this mechanism, because a point mutation in this residue abrogates the inhibitory activity of overexpressed Sprys and even enhances MAPK activation through a dominant-negative mechanism (31, 35, 49). In contrast, in VEGF signaling, which delivers signals through the PLCγ1/protein kinase C pathway to Raf1, independent of Ras, Spry4 directly binds to Raf1 and blocks its phosphorylation, preventing activation (50). This binding is mediated by the C-terminal portion of Spry4, called the cysteine-rich domain. Furthermore, a Tyr<sup>53</sup> mutant of Spry4 still efficiently inhibited VEGF-induced MAPK activation, indicating that this tyrosine residue does not contribute to its action. In contrast, Tyr<sup>53</sup> of Spry4 is essential for inhibition of FGF-induced MAPK activation in the same cells (293T cells), implying that it uses Grb2-SOS sequestration for this action. Therefore, Sprys can use separate binding domains and target proteins to inhibit MAPK activation induced by different growth factors even in the same cells.

Even for the same growth factor signaling, the mode of action or the functional outcome can be different in various cell types. In FGF signaling, Spry1 and 2 act at the Grb2-SOS level in a murine myoblast cell line (C2C12) as described above (35). However, there have been reports that Spry2 inhibits FGF-induced MAPK activation at the Raf level in a human epithelial cell line (293T) and at the Ras level in a murine fibroblast cell line (NIH3T3) (29, 51). For EGF signaling, Spry1 and/or Spry2 inhibited EGF-induced MAPK activation in mouse embryonic fibroblasts (49) and Elk1 activation in a murine myoblast cell line (C2C12) (35). However, in various cell lines, such as epithelial cell lines (HeLa, 293T and CHO) (36, 37, 52) and a human endothelial cell line (HUVEC) (30), Spry1 and 2 either do not inhibit, or enhance EGF-induced MAPK activation. The mechanism underlying this is discussed later.

Lastly, Sprys do not act on the MAPK pathway exclusively. Even though a few papers reported that Spry1 and 2 do not affect JNK, p38, and PI3K/AKT (29, 51), overexpression of *Xenopus* Spry2 inhibited FGF-dependent calcium signaling in *Xenopus* oocytes (53). In HUVEC cells, Spry1 and 2 cannot inhibit EGF-induced proliferation, implying that they can affect signaling pathways other than the MAPK pathway (30). Thus, even though Spry was initially thought to be a simple inhibitor of the Ras/MAPK pathway, it is now clear that these proteins play various roles in RTK signaling using multiple domains of interaction and multiple binding partners. The function of each Spry isoform and its mode of action can vary depending on the cell type and the context of the signaling. Therefore, the role of Spry(s) needs to be carefully examined in each specific cell type and signaling context.

Our data from Th1 clones confirms the previous results for RTK signaling in other cell types that Spry1 can act as a negative regulator. Overexpression of Spry1 in resting Th1 T cell clones using TAT-fusion protein transduction led to decreased IL-2 production following TCR stimulation. In addition, reduction of Spry1 expression by specific siRNA transfection had the opposite effect, increasing IL-2 production in the A.E7 T cell clones. One preliminary experiment using an IL-2 promoter-driven luciferase assay showed that Spry1 overexpression inhibited at the transcriptional level (data not shown).

Consistent with this functional outcome, Spry1 overexpression inhibited TCR-mediated MAPK activation. However, several pieces of evidence suggest that it may use a different biochemical mechanism from that reported in other cells. An overexpressed Tyr53 mutant of Spry1 could still inhibit IL-2 production and MAPK activation as efficiently as, if not better than, the wild-type Spry1. This means Spry1 in T cell clones may not work at the level of Grb2 even though the Grb2-SOS-MAPK pathway is well established in TCR signaling. This idea was further supported by the observation that Spry1 inhibited NF-AT activation, which is a component of the calcium signaling pathway in T cells. As described above, *Xenopus* Spry2 could inhibit FGF-dependent calcium signaling in *Xenopus* oocytes (53). However, *Xenopus* Spry2 did not inhibit the Ras/MAPK pathway at the same time and thus its effect may be different from what we have observed in mouse T cells.

In TCR signaling, the Ras/MAPK pathway can be activated in two different ways. TCR engagement leads to Src kinase-induced activation of CD3  $\zeta$ -chain, and recruitment and phosphorylation of Zap70 and LAT in the TCR complex. Grb2 can then bind to phospho-LAT and a Grb2-SOS complex can deliver an activation signal to Ras (54). In contrast, activated LAT also recruits PLC- $\gamma$ 1, which in turn generates IP<sub>3</sub> and DAG. IP<sub>3</sub> induces intracellular calcium mobilization and subsequent activation of the calcineurin/NF-AT axis. DAG can bind Ras-GRP, a guanine-nucleotide-exchange factor, which then binds to and activates Ras (55). Therefore, it is tempting to speculate that Spry1 can target the second Ras-GRP pathway rather than the Grb2-SOS pathway. If so, one could easily explain the dual effect of Spry1 on MAPK and NF-AT by placing the site of action before the bifurcation of TCR signaling into the calcium and Ras pathways, for example, at the level of PLC $\gamma$ 1 or LAT. In any event, Spry1 definitely appears to use a novel mechanism to down-regulate TCR signaling in Th1 cell clones, adding another example to the uniqueness of growth factor signaling inhibition by Spry proteins.

In contrast to its inhibitory activity in T cell clones, Spry1 overexpression enhanced TCR signaling in naive T cells, leading to increased proliferation and cytokine production. Biochemically, it enhanced both MAPK and NF-AT activation, implying that it may act here as well on proximal signaling complexes, before the bifurcation of the calcium and MAPK pathways. A partial knock-down of Spry1 expression by siRNA transfection had the opposite effect, reducing IL-2 production following TCR activation with either anti-CD3 or peptide/MHC presentation. Thus, in normal TCR signaling of naive T cells, Spry1 plays a significant role in augmenting the response. The dichotomy of the effect of Spry1 manipulation in naive T cells vs T cell clones was surprising; however, a similar dichotomy has been described for EGFR signaling by several groups (36, 37, 52). Much attention has been paid to this phenomenon, because it challenges the idea that Sprys are exclusively negative regulators. These other groups reported that Spry1 and 2 do not inhibit, but enhance MAPK activation in various cell lines in response to EGFR signaling (36, 37, 52). The mechanism proposed was that hSpry2 binds to c-Cbl, an E3 ubiquitin ligase, and sequesters it from the EGFR. This prevents the receptor from being degraded by c-Cbl-mediated ubiquitination and thus sustains downstream MAPK activation. Interestingly, hSpry2 uses phosphorylation of the same Tyr<sup>55</sup> for its c-Cbl binding as it does for its negative signaling in the inhibition of FGF-induced MAPK activation (56). In contrast, our data showed that a Tyr<sup>53</sup> mutant of Spry1, which is comparable to the Tyr<sup>55</sup> mutant of Spry2, did not show any difference from the wild-type Spry1 in naive T cells, implying that Spry1 does not use this tyrosine for enhancing TCR

signaling. Furthermore, in naive T cells, Spry1 overexpression did not affect the level of surface TCR at any time from 0 to 8 h after stimulation (data not shown).

However, it has been reported that hSpry2 as well as Spry1 can bind to the Ring-finger domain of c-Cbl without tyrosine phosphorylation (38, 57). Therefore, considering that we used an overexpression system, we cannot formally exclude the possibility that Spry1 also uses inhibition of c-Cbl to enhance TCR signaling in our experimental system. It is noteworthy that c-Cbl can bind phosphorylated Zap70 and promote ubiquitination of  $\zeta$ -chains in T cells (16). Nevertheless, the dichotomy of action of Spry1 in Th1 cell clones and naive T cells under the same conditions of overexpression as well as following down-regulation by sequence-specific siRNA transfection clearly shows that the role of Spry1 can be different in the same cell type and receptor signaling pathway when the cell is in different states of differentiation. Consistent with this idea, the enhancing effect of Spry1 was abrogated in previously activated T cells. As a mature naive T cell becomes activated by Ag, it experiences changes in many ways, including proliferation as well as differentiation. The resultant preactivated cells show an effector/memory phenotype including new cell surface markers, such as CD44, and enhanced sensitivity to Ag stimulation (58). The protein interactions of its signaling molecules also appear to change somewhat during this differentiation process. For example, in naive T cells, Cbl-b interacts with PI3K downstream of CD28 and prevents hyperphosphorylation of Vav without affecting PLC $\gamma$ 1 (59–61). In contrast, in preactivated T cells, Cbl-b is able to bind to PLC $\gamma$ 1, inhibiting its activation (62). Another example is the responsiveness of T cells to TGF- $\beta$ . TGF- $\beta$  can inhibit proliferation of naive CD4<sup>+</sup> T cells, but cannot do so for preactivated CD4<sup>+</sup> T cells (63). Therefore, it is possible that the target molecules of overexpressed Spry1 change during the differentiation process. Alternatively, either the concentration or the activation status of the Sprys or target molecules may differ between the two states. Among the four Spry homologs, Spry3 showed a different expression pattern in naive T cells (low), compared with the A.E7 T cell clone (high). The level of Spry3 in preactivated T cells was similar to that of the clone (data not shown). Thus, it is possible that the enhancement of Spry3 expression following preactivation reverses the function of Spry1, e.g., formation of a heterodimer (49). So far we have failed to detect a physical interaction between Spry1 and Spry3 by immunoprecipitation experiments; however, further studies are required to evaluate this idea. Finally, the naive T cells, preactivated T cells and Th1 cell clones seem to display a hierarchy of responsiveness to Spry1 overexpression. The more T cells were exposed to Ag, the more susceptible they appeared to negative regulation. This observation suggests that T cells show a gradual change in signaling phenotype depending on their exposure history to Ag. This raises the possibility that the arrangement of proximal signaling complexes in the cytosol might be changing according to the degree of differentiation of the cell.

An increasing number of papers have shown that Sprys have diverse biochemical interactions and functional effects on various RTK-signaling pathways. Intriguingly, each Spry isoform uses separate mechanisms of action for different growth factor signaling in a particular cell type. In some cases, a certain Spry isoform shows different effects in different cell types even for the same growth factor signaling pathway. Our data that Spry1 has a bipolar effect on TCR signaling depending on the differentiation status of T cells is another example of this complexity, emphasizing the multiple roles of Spry1 on the same receptor signaling pathway. The observation that Spry1 affects both the MAPK and NF-AT pathways at the same time in either Th1

clones or naive T cells is the first demonstration that Sprys can affect more than one pathway simultaneously, opening up the possibility of finding novel binding proteins that are unique to T cells. More details of these molecular interactions will eventually provide insight into the different phenotypes observed in naive T cells and Th1 clones.

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## Disclosures

The authors have no financial conflict of interest.

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