

# SOCS-6 Negatively Regulates T Cell Activation through Targeting p56<sup>lck</sup> to Proteasomal Degradation\*

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The T cell-specific tyrosine kinase, p56<sup>lck</sup>, plays crucial roles in T cell receptor (TCR)-mediated T cell activation. Here, we report that SOCS-6 (suppressor of cytokine signaling-6) is a negative regulator of p56<sup>lck</sup>. SOCS-6 was identified as a protein binding to the kinase domain of p56<sup>lck</sup> through yeast two-hybrid screening. SOCS-6 bound specifically to p56<sup>lck</sup> (F505), which mimics the active form of p56<sup>lck</sup>, but not to wild type p56<sup>lck</sup>. In Jurkat T cells, SOCS-6 binding to p56<sup>lck</sup> was detected 1–2 h after TCR stimulation. Confocal microscopy showed that upon APC-T cell conjugation, SOCS-6 was recruited to the immunological synapse and colocalized with the active form of p56<sup>lck</sup>. SOCS-6 promoted p56<sup>lck</sup> ubiquitination and its subsequent targeting to the proteasome. Moreover, SOCS-6 overexpression led to repression of TCR-dependent interleukin-2 promoter activity. These results establish that SOCS-6 acts as a negative regulator of T cell activation by promoting ubiquitin-dependent proteolysis.

The T lymphocyte-specific member of the Src-type tyrosine kinase family, p56<sup>lck</sup>, plays essential roles in development, antigen-induced T cell activation, and proliferation (1–3). During TCR<sup>4</sup>-mediated T cell activation, p56<sup>lck</sup> is activated and transmits a positive signal by interacting with CD4/CD8 (4, 5). Upon engagement of the antigen-major histocompatibility complex with TCR-CD4-CD8 complexes, p56<sup>lck</sup> phosphorylates the immunoreceptor tyrosine-based activation motifs of the CD3  $\xi$ -chain and provides the binding site for ZAP-70 tyrosine kinase (6). Subsequent ZAP-70 activation leads to phosphorylation of signaling proteins, including LAT, and amplification of TCR-mediated signaling (7, 8). In this process, p56<sup>lck</sup> migrates

to T cell-APC contact regions known as immunological synapses (IS) and thereby enhances TCR signaling (9, 10). Given the importance of p56<sup>lck</sup> function in T cell activation, establishing how p56<sup>lck</sup> activity is regulated is essential to understand that activation.

The structure of p56<sup>lck</sup> is similar to that of other Src family kinases and exhibits the following common domains: N-terminal attachment sites for saturated fatty acid addition, a unique region; an Src homology 3 (SH3) domain; an SH2 domain; a tyrosine kinase domain (SH1); and a C-terminal negative regulatory site, tyrosine 505 (Tyr-505) (11). p56<sup>lck</sup> kinase activity is regulated by intramolecular interactions through its regulatory domains and/or phosphorylation (11). For example, phosphorylation of Tyr-394 in the activation loop of the kinase domain increases enzymatic activity, whereas phosphorylation of Tyr-505 renders the enzyme less active. Phosphorylated Tyr-505 interacts with its own SH2 domain, promoting a closed conformation and maintaining the kinase in an inactive state (12). Phenylalanine substitution of the regulatory tyrosine residue (Y505F) results in a constitutively active form of the protein. The status of the regulatory tyrosine is maintained both by a kinase, Csk, which phosphorylates it (13), and by a transmembrane phosphatase, CD45, which dephosphorylates it (14). In addition, p56<sup>lck</sup> kinase activity is regulated through intramolecular interaction of the SH3 domain with a proline-rich motif in the SH2-kinase linker region. This interaction maintains a conformation in which the kinase domain is inaccessible (15).

In addition to intramolecular interaction, protein stability of active p56<sup>lck</sup> also regulates enzymatic activity. Upon T cell activation, c-Cbl interacts with the SH3 domain of p56<sup>lck</sup> through its proline-rich motif and then depletes p56<sup>lck</sup> from plasma membrane lipid rafts (16). Depleted p56<sup>lck</sup> is ubiquitinated by c-Cbl and subsequently degraded by the proteasome upon T cell activation (17). On the other hand, heat shock protein 90 (Hsp90) prevents active p56<sup>lck</sup> from being targeted for degradation by ubiquitination (18–20). Recent work also suggests that TCR-induced ubiquitination occurs at the IS (21). During TCR stimulation, ubiquitinated proteins accumulate at the T cell-APC contact site (21).

In this study, we identified SOCS-6 (suppressor of cytokine signaling-6) as a protein binding to the kinase domain of p56<sup>lck</sup>. SOCS-6 is a member of eight SOCS family proteins, including cytokine-inducible SH2-containing protein (CIS) and SOCS-1–7 (22–25). SOCS proteins contain N termini of various lengths and sequence, an SH2 domain, and a C-terminal SOCS box. The model for activity of SOCS proteins, which is based

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<sup>4</sup> The abbreviations used are: TCR, T cell receptor; SOCS, suppressor of cytokine signaling; IS, immunological synapse; SH2, Src homology 2; IL-2, interleukin-2; CIS, cytokine-inducible SH2-containing protein; X-gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; GST, glutathione S-transferase; KID, kinase-interacting domain; aa, amino acid; HA, hemagglutinin; siRNA, small interfering RNA; PMA, phorbol 12-myristate 13-acetate; SEE, *Staphylococcus enterotoxin E*; wt, wild type; LLnL, *N*-acetyl-Leu-Leu-norleucinal; APC, antigen-presenting cell.

## SOCS-6 as a Negative Regulator of T Cell Activation

primarily on studies of CIS and SOCS-1–3, is that upon cytokine binding SOCS genes are rapidly up-regulated, and their protein products block further signaling by inactivating JAK-STAT pathways or targeting binding partners to E3 ubiquitin ligases, thus blunting cytokine responses (26). SOCS proteins are connected to the E3 ubiquitin-ligase complex via interaction of SOCS box with elongin BC (27, 28). The elongin BC complex associates with members of the cullin family and Rbx1 to form an E3 ubiquitin-ligase complex (29). Unlike other SOCS family members, such as SOCS-1–3 and CIS, relatively little is known about functions of SOCS-4–7. SOCS-6 does not interact with molecules in the JAK-STAT pathway or inhibit cytokine signaling (30). However, SOCS-6 has been shown to inhibit insulin and stem cell factor signaling (30, 31) by interacting with the p85 subunit of phosphatidylinositol 3-kinase as well as with IRS-2 and IRS-4, although controversial data have emerged from animal studies using SOCS-6 null or transgenic mice, which show normal or positive responses to insulin, respectively (31, 32).

Here, through yeast two-hybrid screening using p56<sup>lck</sup> as bait, we found that SOCS-6 associated with p56<sup>lck</sup> and targeted it to proteasomal degradation by promoting its ubiquitination. In response to TCR stimulation, SOCS-6 was recruited to the immunological synapse and colocalized with the active form of p56<sup>lck</sup>. Furthermore, SOCS-6 overexpression inhibited TCR-dependent IL-2 promoter activation. These results provide a novel inhibitory mechanism of T cell activation operating at the level of stability of active p56<sup>lck</sup> protein.

### EXPERIMENTAL PROCEDURES

**Yeast Two-hybrid Screening**—Screening was performed as described previously (33). The bait plasmid, pGBT9-p56<sup>lck</sup> SH2-kinase (F505), encodes the SH2 and kinase domains of p56<sup>lck</sup>. The 2 × 10<sup>6</sup> transformants of a mouse T cell lymphoma cDNA library (BD Biosciences) were screened. Isolation of true positive clones and determination of binding specificity were performed as described previously (34).

**Plasmids**—Full-length SOCS-6 cDNA was obtained by rapid amplification of cDNA ends using the mouse T cell lymphoma cDNA library as template and cloned into the XhoI and BamHI sites of pcDNA3.1 (Invitrogen). EF/BOS plasmids expressing SOCS-1, SOCS-2, SOCS-3, and CIS were obtained from Dr. Tracy A. Willson (35). As baits for the yeast two-hybrid assay, corresponding DNA fragments were amplified by PCR with Pyrobest enzyme (Takara) using primers with EcoRI (5'-primer) or BamHI (3'-primer) site extension. Amplified regions include p56<sup>lck</sup> kinase (amino acids (aa) 225–509), p56<sup>lck</sup> SH2-kinase (aa 123–509), p56<sup>lck</sup> SH2 (aa 123–225), p60<sup>src</sup> SH2-kinase (aa 150–533), p59<sup>lyn</sup> SH2-kinase (aa 143–534), Jak1 kinase (aa 855–1152), Tyk2 kinase (aa 897–1176), Itk SH2-kinase (aa 239–615), ZAP-70 kinase (aa 338–600), Abl SH2-kinase (aa 112–493), platelet-derived growth factor receptor kinase (aa 560–1014), and epidermal growth factor receptor kinase (aa 1018–1210) domains. After digestion with EcoRI and BamHI, fragments were inserted into corresponding sites of pGBT9 (BD Biosciences). As prey, DNA fragments encoding SOCS-6, SOCS-1, SOCS-2, and p56<sup>lck</sup> SH2-kinase domain were amplified by PCR and inserted into the EcoRI and BamHI sites of

pGAD424 (BD Biosciences) using the method described above. For mapping the binding domain using the two-hybrid system, fragments encoding N- or C-terminal deletion mutants of SOCS-6 were generated by PCR and inserted into the EcoRI and BamHI sites of pGBT9. For *in vitro* transcription/translation or for mammalian cell expression, DNA fragments encoding Myc/His-tagged SOCS-6 or deleted forms of SOCS-6 were cloned into the XhoI and BamHI sites of pcDNA 3.1 Myc-His (Invitrogen). Internal deletion mutants, SOCS-6 ΔSH2 and ΔKID, were generated by mutagenesis using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA). To generate GST fusion proteins, a DNA fragment encompassing the p56<sup>lck</sup> kinase domain was inserted into the BamHI and XhoI sites of pGEX4T-2 (GE Healthcare). The pMT123 plasmid coding for the hemagglutinin (HA)-tagged ubiquitin (36), the reporter plasmid, IL-2-Luc (37), and expression plasmids for the p56<sup>lck</sup> wild type (WT) or F505 (38) are described elsewhere.

The pSUPER plasmid (39) expressing siRNA was produced as described previously (38). Three different 19-nucleotide gene-specific sequences corresponding to 592–610 (TGGAGACCTGCATCTTCAC), 704–722 (GGATCAGGAGCTATTGTC), and 1346–1364 (GGACATACGTC-CATAGTTG) in the human SOCS-6 coding region were selected as targets to construct siRNA-expressing vectors. The construct expressing nucleotides 704–722 was determined as effective in reducing SOCS-6 levels and used for the remaining experiments. For control siRNA of nucleotides 704–722, the sequence was changed at the two underlined nucleotides, GGATCACCGAGCTATTCT.

**GST Pulldown Assay**—GST or GST-p56<sup>lck</sup> kinase domain was expressed in *Escherichia coli* BL21 and immobilized on glutathione-Sepharose 4B beads. SOCS-6 and deletion mutants were synthesized in the presence of [<sup>35</sup>S]methionine using TNT T7-quick coupled transcription/translation kit (Promega). <sup>35</sup>S-Labeled proteins were mixed with immobilized GST or GST fusion protein in 1 ml of binding buffer (phosphate-buffered saline containing 0.25% Nonidet P-40) and incubated at 4 °C for 1 h. After five washes with 1 ml binding buffer, bound proteins were eluted with SDS sample buffer, separated on 4–20% SDS-PAGE, and analyzed by autoradiography.

**Antibodies and Chemicals**—Polyclonal anti-SOCS-6 serum was generated by immunizing rabbits with GST-SOCS-6 fusion protein purified by affinity chromatography using glutathione-Sepharose 4B beads (GE Healthcare). Polyclonal anti-p56<sup>lck</sup> serum was also generated using the same method (34). OKT3 antibody was previously described (37). To precipitate p56<sup>lck</sup>, anti-p56<sup>lck</sup> antibody conjugated to beads (Santa Cruz Biotechnology) or anti-p56<sup>lck</sup> antibody (Upstate) was employed. Other antibodies were obtained from commercial sources and include the following: anti-SOCS-6, anti-CIS, anti-SOCS-1, anti-HA, anti-ubiquitin, and anti-His tag (Santa Cruz Biotechnology); anti-CD3 and anti-CD28 (UCHT-1) (BD Biosciences); anti-phospho-Src (Tyr-417) (Cell Signaling Technology, Beverly, MA); anti-actin (Sigma), and Alexa 568- or 488-conjugated secondary antibodies (Invitrogen). Proteasome inhibitors, *N*-acetyl-Leu-Leu-norleucinal (LLnL) and epoxomicin (Sigma), and *Staphylococcus* enterotoxin E (SEE) (Toxin Technology, Sarasota, FL) were also used.

**Transfection and T Cell Activation**—Transfection and T cell activation were performed as described previously (34). Briefly, HEK293 cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. At 24 h post-transfection, cells were treated with 100 nM epoxymycin for 6 h. Jurkat T cells were transfected by electroporation (BTX Co., Ltd.). Briefly,  $1.5 \times 10^7$  Jurkat T cells were combined with 20  $\mu$ g of DNA in an electroporation cuvette, pulsed once at 240 V for 25 ms, and incubated overnight at 37 °C for further processing. pEGFP-N1 (BD Biosciences) was included in the DNA mixture to estimate transfection efficiency. We routinely obtained transfection efficiency of greater than 50% as measured by fluorescence-activated cell sorter analysis of green fluorescent protein-expressing cells.

For the *in vivo* binding assay, Jurkat T cells were activated by cross-linking CD3 with the OKT3 monoclonal antibody. Cells were pretreated with the proteasome inhibitor LLnL for 2 h prior to CD3 stimulation.  $5 \times 10^7$  cells were stimulated by incubating them on plates coated with 10  $\mu$ g/ml OKT3 antibody in phosphate-buffered saline at 37 °C for the indicated times. After washing twice with ice-cold phosphate-buffered saline, cells were extracted in TNE buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 5 mM sodium orthovanadate, 5 mM sodium fluoride, and a protease inhibitor mixture) for 1 h on ice. To assay p56<sup>lck</sup> ubiquitination,  $5 \times 10^6$  Jurkat T cells were preincubated with LLnL for 2 h and activated by anti-CD3 cross-linking. Subsequently, cells were stimulated by incubation with 10  $\mu$ g/ml UCHT-1 antibody in phosphate-buffered saline at 37 °C for the indicated times. After washing twice with ice-cold phosphate-buffered saline, cells were extracted in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 5 mM sodium orthovanadate, 5 mM sodium fluoride, *N*-ethylmaleimide, and a protease inhibitor mixture) for 30 min on ice.

**Primary T Cell Activation**—Primary T cells were purified from C57BL/6 mice. Briefly, Thy1.2<sup>+</sup> T cells were isolated from pooled lymph nodes and spleen cells by positive selection using the magnetic-activated cell sorting column (Miltenyi Biotec). Resting T cells were stimulated with anti-CD3 (5  $\mu$ g/ml) and anti-CD28 (2.5  $\mu$ g/ml) antibodies.

**Immunoprecipitation and Western Blot Analysis**—For immunoprecipitation, cell extracts were prepared in TNE buffer and incubated with 40  $\mu$ l of 50% slurry of anti-p56<sup>lck</sup> antibody-conjugated beads overnight at 4 °C. The precipitated p56<sup>lck</sup>-immune complex was washed five times with TNE or NETN buffer. For Western blot analysis, immune complexes or cell extracts were separated on SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with appropriate antibodies. Bands were detected using the Advanced ECL reagent (Amersham Biosciences).

**Confocal Microscopy**—Jurkat T cells were cotransfected with expression plasmids encoding Myc-SOCS-6 and p56<sup>lck</sup> (Tyr-505). T cell-APC conjugates were then formed using Raji B cells as APC (34). Prior to conjugation, Raji B cells were incubated with 5  $\mu$ g/ml SEE at 37 °C for 1 h. Transfected Jurkat T cells and Raji B cells were mixed 1:1, immediately transferred to poly-L-lysine-coated slides, and incubated at 37 °C for the indicated times. Slides were fixed in 3.7% formaldehyde for 20 min, per-

meabilized in 0.1% Triton X-100 for 4 min at room temperature, and incubated with indicated antibodies in 3% bovine serum albumin for 30 min at 30 °C. Subsequently, slides were exposed to Alexa 568- or Alexa 488-coupled secondary antibodies (Invitrogen) and incubated for 30 min at 30 °C. After mounting, photographs were taken using a LSM510 META confocal microscope equipped with an Axiovert  $\times 100$  1.4 oil immersion objective lens (Carl Zeiss, Oberkochen, Germany). Cross-talk was resolved through multitracking of the laser source. Images were automatically assessed for distribution of conjugates along a single two-dimensional optical section (along the *x-y* axis). The degree of colocalization of two molecules was analyzed by using LSM510 META software. All pixels with the same position in both images were considered coincidental, and the brightness level of each pixel was plotted on the *x* and *y* axes. Pearson's correlation coefficient ( $R_p$ ) provides information on the intensity distribution within the colocalizing region.  $R_p$  value of 1.0 represents 100% colocalization. Conjugates with  $R_p$  value  $>0.5$  was considered as conjugates with colocalization.

**Reporter Assay**—For the IL-2-firefly luciferase (IL-2-Luc) reporter assay, Jurkat cells were transfected with 5  $\mu$ g each of IL-2-Luc along with indicated amounts of plasmids expressing SOCS-6, SOCS-1, SOCS-2, SOCS-3, or CIS using Superfect<sup>TM</sup> (Qiagen). The total amount of DNA was kept constant by adjusting with empty vector plus 1 or 10 ng of pTK-R-Luc (*Renilla* luciferase; Promega, Madison, WI) to normalize transfection efficiency. At 24 h post-transfection, cells were stimulated by incubating on OKT3-coated plates or by treatment with 5 ng/ml phorbol 12-myristate 13-acetate (PMA) plus 500 ng/ml A23187 for 12 h at 37 °C, and then extracted with Passive Lysis Buffer (Promega). Firefly and *Renilla* luciferase activities were assessed using a Dual-Luciferase reporter assay kit (Promega).

## RESULTS

**Identification of SOCS-6 as a Binding Partner of the p56<sup>lck</sup> Kinase Domain**—To understand the regulatory mechanism of p56<sup>lck</sup>, we performed a yeast two-hybrid screening of a T cell lymphoma cDNA library using the activated F505 mutant form of p56<sup>lck</sup> SH2-kinase as bait (34, 37). Partial nucleotide sequencing of positives identified four clones encoding parts of SOCS-6. Binding of SOCS-6 to p56<sup>lck</sup> was further analyzed in additional yeast two-hybrid assays (Fig. 1). SOCS-6 interacted with the p56<sup>lck</sup> kinase domain but not with the SH2 domain. SOCS-6 binding was not affected by introduction of a mutation in the autophosphorylation site (the p56<sup>lck</sup> SH2-kinase (Ala-273) mutant, a catalytically inactive form), indicating that association of p56<sup>lck</sup> with SOCS-6 does not require kinase activity. Binding specificity of SOCS-family proteins, SOCS-1, 2, and 6, to various protein-tyrosine kinases was analyzed using combinations of baits and preys shown in Fig. 1. Interestingly, SOCS-6 more readily bound to the SH2-kinase domain of p56<sup>lck</sup> (F505) than to that of p60<sup>src</sup> (Phe-527) and p59<sup>lyn</sup> (Phe-528). Moreover, SOCS-6 did not interact with kinase domains of other protein-tyrosine kinases examined, namely Abl, Itk, ZAP-70, Jak, Tyk2, platelet-derived growth factor receptor, and epidermal growth factor receptor, indicating specificity of the inter-

|                                      | β-gal assay |        |        |
|--------------------------------------|-------------|--------|--------|
|                                      | SOCS-6      | SOCS-1 | SOCS-2 |
| p56 <sup>lck</sup> SH2-Kinase (F505) | ***         | -      | -      |
| p56 <sup>lck</sup> SH2-Kinase (A273) | ***         | -      | -      |
| p56 <sup>lck</sup> SH2               | -           | -      | -      |
| p56 <sup>lck</sup> Kinase            | **          | -      | -      |
| p60 <sup>src</sup> SH2-Kinase (F527) | *           | -      | -      |
| p59 <sup>lyn</sup> SH2-Kinase (F528) | -           | -      | -      |
| Abl SH2-Kinase                       | -           | -      | -      |
| Itk SH2-kinase                       | -           | -      | -      |
| ZAP-70 kinase                        | -           | -      | -      |
| Jak1 kinase                          | -           | ***    | -      |
| Tyk2 kinase                          | -           | ***    | -      |
| PDGFR kinase                         | -           | -      | ***    |
| EGFR kinase                          | -           | -      | -      |

FIGURE 1. SOCS-6 specifically associates with the kinase domain of p56<sup>lck</sup> in a yeast two-hybrid system. Full-length cDNAs encoding SOCS-1, SOCS-2, and SOCS-6 were fused to the GAL4 activation domain, whereas the SH2-kinase or kinase domain of various protein-tyrosine kinases was fused to GAL4 BD. Five days after cotransformation, a β-galactosidase (β-gal) assay was performed. Binding affinity was scored as \*\*\* (deep blue), \*\* (intermediate blue), \* (pale blue), or - (white) after X-gal staining. PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor.

action between SOCS-6 and p56<sup>lck</sup>. As reported previously (25), we found that SOCS-1 and SOCS-2 interacted with Jak1/Tyk2 and the platelet-derived growth factor receptor, respectively, further supporting specificity of the assay (Fig. 1).

**Mapping the Binding Domain**—Next, we determined the SOCS-6 domain required for interaction with p56<sup>lck</sup> using SOCS-6 deletion mutants (Fig. 2A). SOCS-6 consists of a long, unique N terminus, an SH2 domain, and a SOCS box. Interestingly, we observed that the N terminus only, but not the SH2 or SOCS box domain, bound to the p56<sup>lck</sup> kinase domain, and through subsequent domain analysis, we found that a region between aa 47 and 218, thereafter designated the kinase-interacting domain (KID), participates in the interaction.

To confirm results shown in Fig. 2A, a set of SOCS-6 deletion mutants shown in Fig. 2B was tested for binding in a GST pull-down assay. *In vitro* translated and radiolabeled SOCS-6 WT, N, ΔSB, and ΔSH2, all of which contain the N terminus, bound to the GST-p56<sup>lck</sup> kinase domain but not to GST only. However, SOCS-6 ΔN and ΔKID did not bind to GST-p56<sup>lck</sup> kinase indicating that KID is essential for binding (Fig. 2C). These results establish p56<sup>lck</sup> as the first molecule interacting with a unique N-terminal region of SOCS-6. In addition, they explain why a previous report did not detect an interaction between p56<sup>lck</sup> and SOCS-6 in a yeast two-hybrid assay because the SOCS-6 construct employed as bait in that study lacked the N-terminal region (25).

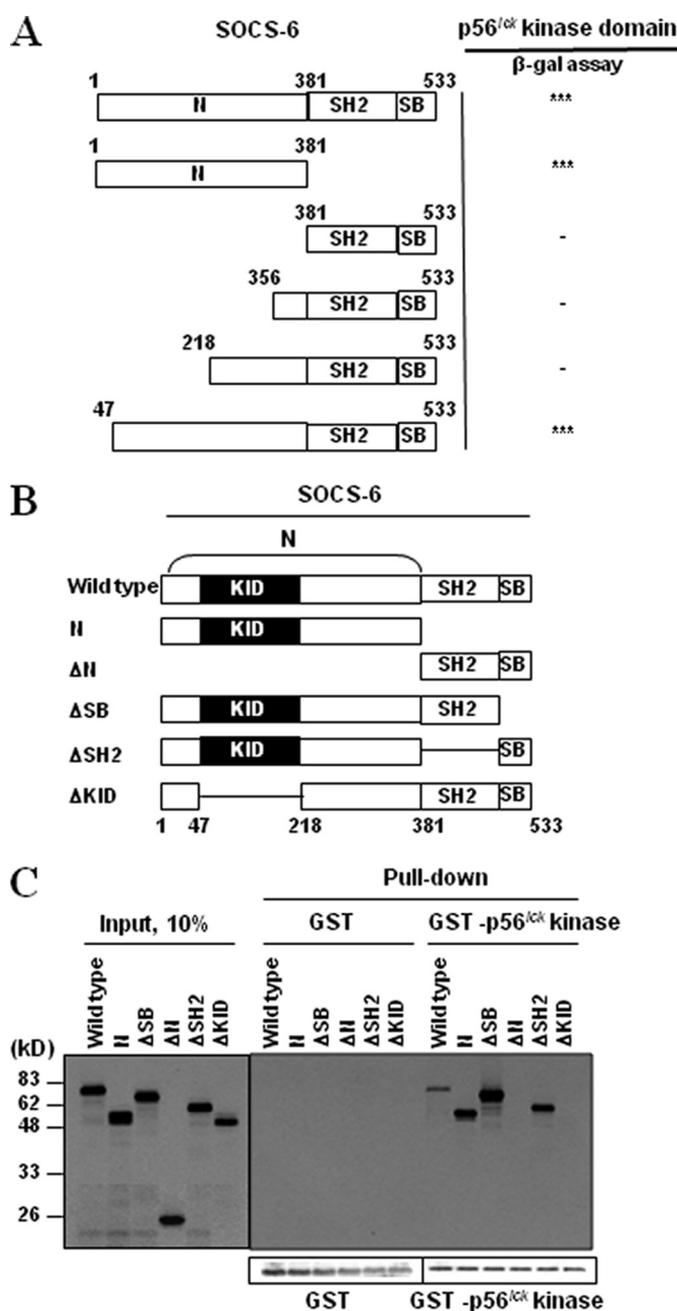


FIGURE 2. Determination of the p56<sup>lck</sup>-binding site on SOCS-6. A, indicated deletion mutants of SOCS-6 were fused to GAL4 activation domain and coexpressed with the GAL4 DNA binding domain-p56<sup>lck</sup> kinase domain in SFY526 cells. N, SH2, and SB represent N terminus, SH2 domain, and SOCS box, respectively. Binding affinity was scored as \*\*\* (deep blue), \*\* (intermediate blue), \* (pale blue), or - (white) after X-gal staining. β-gal, β-galactosidase. B, schematic diagram of SOCS-6 deletion mutants used for *in vitro* binding site mapping. N, N-terminal region; ΔN, lacking N-terminal; ΔSB, lacking SOCS box; ΔSH2, lacking SH2 domain; and ΔKID, lacking aa 47–218. C, pull-down assay of radiolabeled SOCS-6 deletion mutants is described under "Experimental Procedures." KID is essential for interaction of SOCS-6 with the p56<sup>lck</sup> kinase domain. As a control, a part of the radiolabeled input was analyzed by fluorography (left panel). Purified GST fusion proteins were visualized by silver staining (lower panel).

**SOCS-6 and p56<sup>lck</sup> Associate *In Vivo* in Response to TCR Stimulation**—Next, we asked whether SOCS-6 binds p56<sup>lck</sup> in mammalian cells. HEK293 cells were cotransfected with plasmids expressing SOCS-6 and p56<sup>lck</sup> (WT or F505). Because SOCS proteins are known to target interacting proteins for proteasomal degradation, we treated cells for 6 h with epoxomicin,

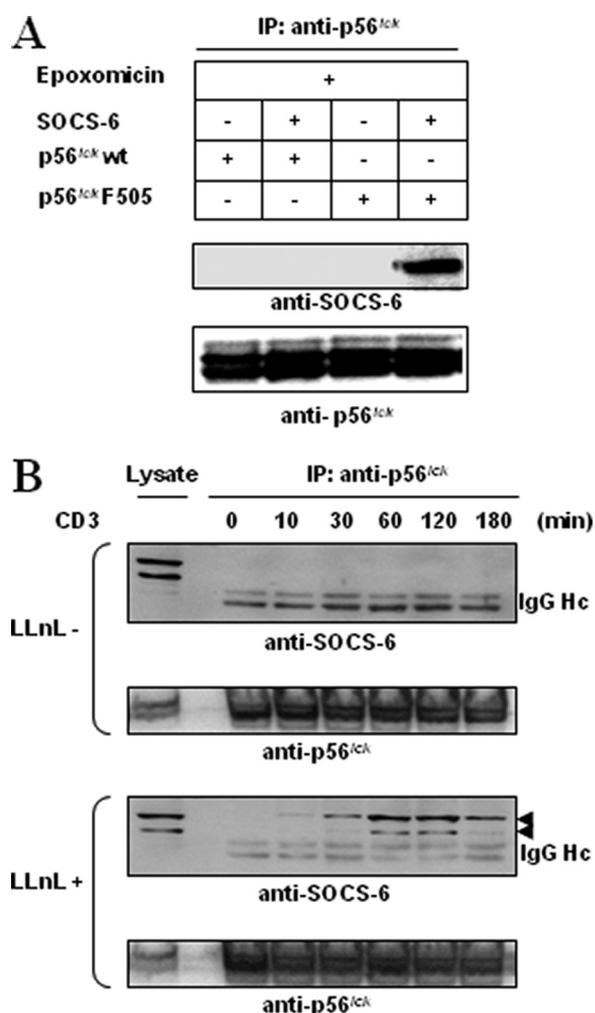


FIGURE 3. *In vivo* binding of SOCS-6 and p56<sup>lck</sup> in T cells. **A**, SOCS-6 binds to p56<sup>lck</sup> (F505), but not p56<sup>lck</sup> (wt), in the presence of a proteasome inhibitor. SOCS-6 was coexpressed with p56<sup>lck</sup> (wild type or F505) by transient transfection of HEK293 cells. Twenty four hours later, cells were treated for 6 h with 100 nM epoxomicin, and then cell extracts were analyzed by immunoprecipitation (IP) with an anti-p56<sup>lck</sup> antibody and immunoblotting with SOCS-6 (upper panel) or p56<sup>lck</sup> (lower panel) antibodies. **B**, in response to TCR stimulation, SOCS-6 associates with p56<sup>lck</sup>. Jurkat T cells were stimulated by CD3 cross-linking for 0, 10, 30, 60, 120, and 180 min in the absence (upper panel) or presence (lower panel) of 100 nM LLnL. Cell extracts were analyzed by precipitation with anti-p56<sup>lck</sup> antibody beads and subsequent immunoblotting with p56<sup>lck</sup> or SOCS-6 antibodies. Arrowheads indicate the position of SOCS-6.

an irreversible proteasome inhibitor. In subsequent coimmunoprecipitations, SOCS-6 protein was detected in the p56<sup>lck</sup> (F505) immunoprecipitates but not in the p56<sup>lck</sup> (WT) immunoprecipitates, indicating that SOCS-6 specifically interacts with the active (F505) form of p56<sup>lck</sup> (Fig. 3A).

Next, we asked whether endogenous p56<sup>lck</sup> bound to SOCS-6 following T cell activation. Jurkat T cells were stimulated by CD3 cross-linking, and after 0, 10, 30, 60, 120, and 180 min, interaction between p56<sup>lck</sup> and SOCS-6 was analyzed by coimmunoprecipitation (Fig. 3B). Interestingly, SOCS-6 was detected in p56<sup>lck</sup> immunoprecipitates starting from 10 min with maximum binding seen at 60 and 120 min after T cell activation but only in the presence of the proteasome inhibitor LLnL (Fig. 3B). Taken together, these results show that SOCS-6 interacts with active p56<sup>lck</sup> and targets active p56<sup>lck</sup> to proteasomal degradation 60–120 min after T cell activation.

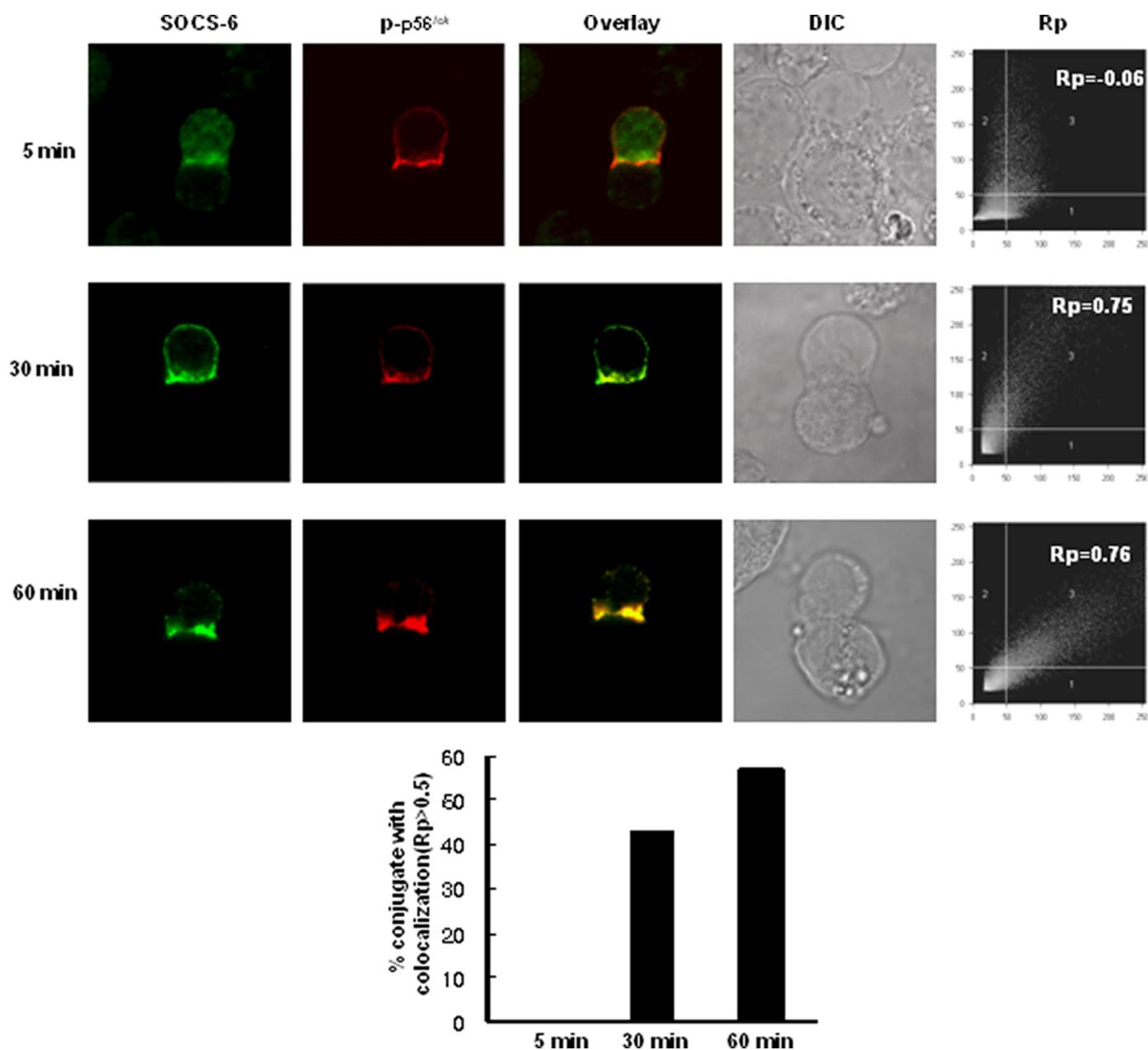
**SOCS-6 Colocalizes with Tyrosine-phosphorylated p56<sup>lck</sup> at the Immunological Synapse**—When T cells encounter APCs, p56<sup>lck</sup> is recruited to sites of T cell-APC contact. Because SOCS-6 coimmunoprecipitates with p56<sup>lck</sup> (F505) following T cell activation, we analyzed localization of these proteins following T cell-APC conjugation. Jurkat T cells were transfected with constructs expressing p56<sup>lck</sup> and Myc-tagged SOCS-6 and were allowed to conjugate with SEE-pulsed Raji B cells. Cells were then stained with anti-Myc or anti-phospho-Lck antibodies, the latter detecting the active form of p56<sup>lck</sup> with tyrosine 394 phosphorylated in the activation loop, and analyzed by confocal microscopy (Fig. 4). As reported (10), the phosphorylated form of p56<sup>lck</sup> (Fig. 4, red color) localized to IS at all time points tested. At 5 min after conjugation, SOCS-6 (Fig. 4, green color) was seen throughout the cell, and colocalization was not detectable at the IS, although a portion of SOCS-6 localized to the IS. At 30 min, most SOCS-6 translocated to the IS, and a significant portion colocalized with phosphorylated p56<sup>lck</sup>. At 60 min, SOCS-6 colocalized precisely with phosphorylated p56<sup>lck</sup> at the IS. In addition, we tested the colocalization of ΔKID with p56<sup>lck</sup> (F505) and found that ΔKID also colocalized with p56<sup>lck</sup> (F505) at the IS (data not shown), suggesting that translocation of SOCS-6 to the IS is not dependent on binding to p56<sup>lck</sup>. These observations establish that SOCS-6 is recruited to the IS upon T cell-APC conjugation and also indicate that SOCS-6 associates with the active form of p56<sup>lck</sup> at the IS. The time of maximal colocalization of SOCS-6 and p56<sup>lck</sup>, 60 min after TCR stimulation, coincides with the time at which maximal binding between SOCS-6 and p56<sup>lck</sup> was observed (Fig. 3B).

**SOCS-6 Overexpression Promotes Proteasomal Degradation of the Active Form of p56<sup>lck</sup>**—The fact that binding between SOCS-6 and p56<sup>lck</sup> is detected only in the presence of a proteasome inhibitor (Fig. 3) suggests that SOCS-6 may target p56<sup>lck</sup> to proteasomal degradation. Therefore, the effect of SOCS-6 overexpression on levels of coexpressed p56<sup>lck</sup> (WT) or p56<sup>lck</sup> (F505) was analyzed in HEK293 cells. Upon overexpression of SOCS-6 but not ΔKID, levels of upwardly shifted forms of p56<sup>lck</sup> (F505) but not p56<sup>lck</sup> (WT) were significantly reduced (Fig. 5A), implying that SOCS-6 may selectively target the active form of p56<sup>lck</sup> to the degradation pathway.

Next, we asked whether p56<sup>lck</sup> (F505) degradation requires the proteasomal pathway. HEK293 cells were cotransfected with plasmids expressing SOCS-6 WT or ΔKID plus p56<sup>lck</sup> (WT or F505). Twenty four hours later, cells were treated with epoxomicin for 6 h and analyzed by immunoblotting. In the absence of epoxomicin, p56<sup>lck</sup> (F505) protein levels were reduced in the presence of coexpressed SOCS-6, but not ΔKID, in a dose-dependent manner. However, in the presence of epoxomicin, p56<sup>lck</sup> (F505) degradation was completely blocked (Fig. 5B), indicating that down-regulation of p56<sup>lck</sup> levels by SOCS-6 is mediated by proteasomal degradation. In the same experiment, levels of p56<sup>lck</sup> (WT) and actin proteins remained constant even in the presence of SOCS-6 (Fig. 5B).

Subsequently, we asked whether SOCS-6 functioned in the ubiquitination of the p56<sup>lck</sup> in response to TCR stimulation. In control Jurkat T cells, p56<sup>lck</sup> ubiquitination was not detectable before TCR stimulation but increased in a time-dependent manner 10 and 30 min after TCR stimulation (Fig. 5C, lanes

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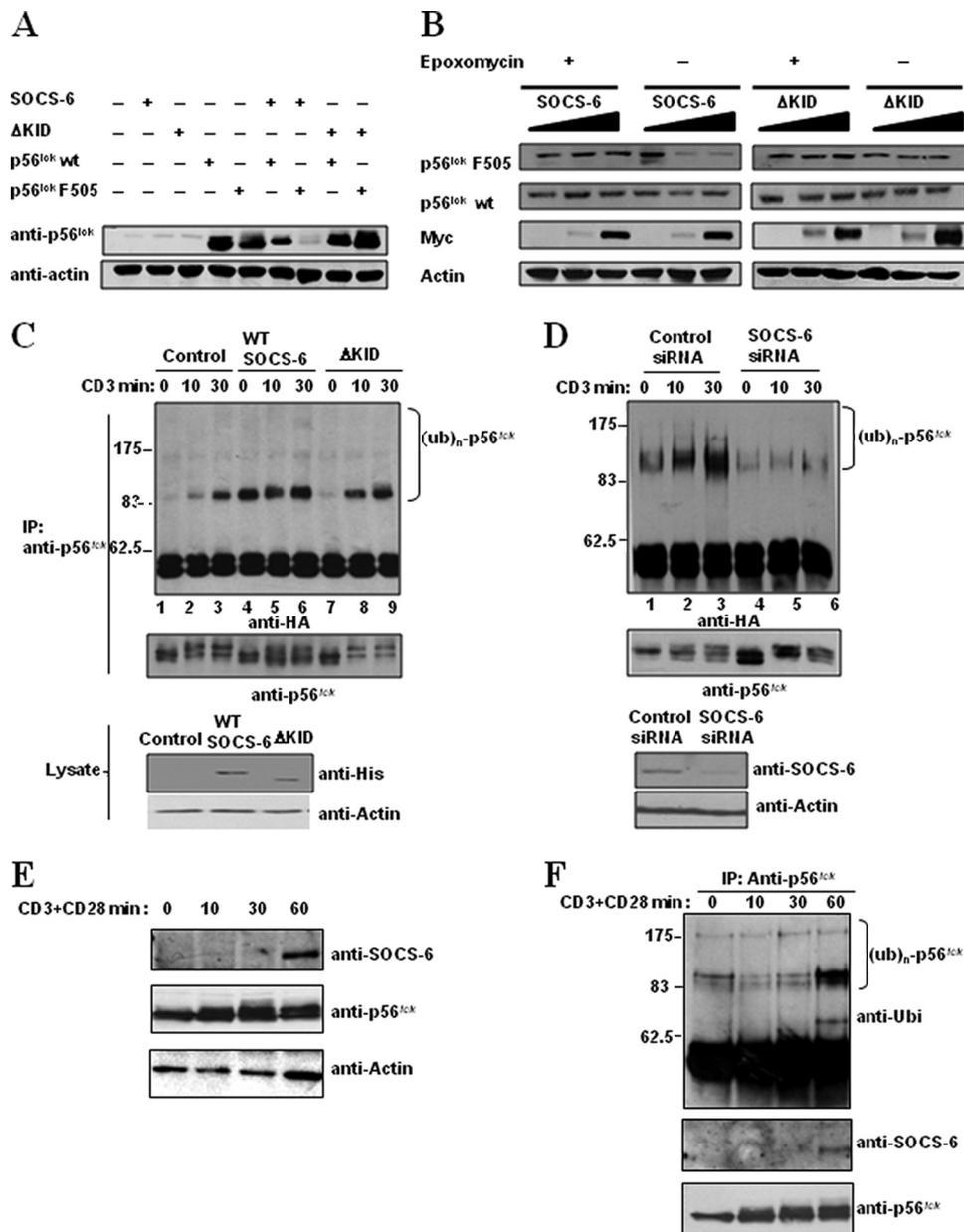
**FIGURE 4. SOCS-6 colocalizes with the active form of p56<sup>lck</sup> at the immunological synapse.** Jurkat T cells were transfected with an expression plasmid encoding Myc-tagged SOCS-6. Cells were then mixed 1:1 with Raji B cells pulsed with SEE (5  $\mu$ g/ml) and allowed to conjugate for 5, 30, and 60 min at 37 °C. Cell conjugates were stained with anti-Myc (9E10) antibody (green, left) and rabbit anti-phospho-Lck (red, middle). A single two-dimensional optical section of the cell conjugate is shown. Untransfected cells or B cells were not detected by antibody. Note that SOCS-6 colocalized with phospho-p56<sup>lck</sup> 60 min after TCR stimulation. The experiments were repeated three times, and the representative conjugates are shown. The degree of colocalization of individual images was quantified by Pearson's correlation coefficient ( $R_p$ ). Out of the total conjugates examined, the percentage of conjugates showing  $R_p$  value >0.5 is depicted in the bottom graph ( $n = 30 =$  total number of conjugates examined for each staining). DIC, differential interference contrast.

1–3). When SOCS-6 was overexpressed, p56<sup>lck</sup> ubiquitination was dramatically enhanced even before TCR stimulation (Fig. 5C, lane 4). Moreover, when stimulated with anti-CD3, enhanced accumulation of ubiquitinated p56<sup>lck</sup> was observed in the presence of SOCS-6 compared with the control (Fig. 5C, compare lanes 5 and 6 with lanes 2 and 3). The  $\Delta$ KID mutant only slightly enhanced p56<sup>lck</sup> ubiquitination (Fig. 5C, lanes 7–9).

To further confirm the role of SOCS-6 in TCR stimulation-dependent p56<sup>lck</sup> ubiquitination, the same assay was performed in Jurkat T cells expressing either control or SOCS-6 siRNA.

After CD3 stimulation, p56<sup>lck</sup> ubiquitination increased in a time-dependent manner in control cells (Fig. 5D, lanes 1–3). In contrast, in cells expressing SOCS-6 siRNA, p56<sup>lck</sup> ubiquitination was almost completely abrogated (Fig. 5D, lanes 4–6). Taken together, these results show that in response to TCR stimulation, SOCS-6 promotes p56<sup>lck</sup> ubiquitination and targets it to the proteasome.

*In Primary T Cells, SOCS-6 Associates with p56<sup>lck</sup> upon T Cell Activation*—We next confirmed the relationship between p56<sup>lck</sup> and SOCS-6 in primary T cells. First, we analyzed whether the expression of SOCS-6 is regulated upon T cell activation.



**FIGURE 5. SOCS-6 promotes proteasomal degradation of the active form of p56<sup>lck</sup>.** A, levels of p56<sup>lck</sup> (F505) are down-regulated by SOCS-6. HEK293 cells were cotransfected with 500 ng of pcDNA3.1/SOCS-6 or ΔKID along with 200 ng of pcDNA/p56<sup>lck</sup> (wt or F505). Cell extracts were analyzed by Western blotting with anti-p56<sup>lck</sup> antibody. B, SOCS-6 promotes proteasomal degradation of p56<sup>lck</sup> (F505) in a dose-dependent manner. HEK293 cells were cotransfected with 0, 200, and 1,000 ng of pcDNA3.1/SOCS-6-His or ΔKID along with 50 ng of pcDNA/p56<sup>lck</sup> (wild type or F505). At 24 h after transfection, cells were treated with 100 nM epoxomicin and then incubated for an additional 6 h. Cell extracts were analyzed by immunoblotting with antibody against p56<sup>lck</sup>, the His tag (to detect SOCS-6), or β-actin. C, SOCS-6 enhances ubiquitination of p56<sup>lck</sup>. Jurkat T cells were cotransfected with plasmids expressing His-tagged SOCS-6 WT or ΔKID with HA-tagged ubiquitin. Twenty-four h later, cells were treated with LLNL for 2 h and subsequently stimulated with anti-CD3 antibody for 0, 10, and 30 min. Cell lysates were subjected to immunoprecipitation (IP) with anti-p56<sup>lck</sup> antibody and immunoblotting with anti-HA antibody. As a control, cell lysates were immunoblotted with anti-His and anti-actin antibodies. D, SOCS-6 siRNA decreases ubiquitination of p56<sup>lck</sup>. Jurkat T cells were cotransfected with plasmids expressing SOCS-6 siRNA or control siRNA plus plasmid encoding HA-tagged ubiquitin (*ub*). Two days later, cells were treated with LLNL for 2 h and stimulated with anti-CD3 antibody for 0, 10, and 30 min. Cell extracts were subjected to immunoprecipitation with anti-p56<sup>lck</sup> antibody and immunoblotting with anti-HA and anti-p56<sup>lck</sup> antibodies. As a control, cell extracts were immunoblotted with anti-SOCS-6 and anti-actin antibodies. E, SOCS-6 expression is up-regulated upon T cell activation. Primary T cells were stimulated with anti-CD3 and CD28 antibodies for indicated times. Cell lysates were subjected to immunoblotting with anti-SOCS-6, anti-p56<sup>lck</sup>, and anti-actin antibodies. F, SOCS-6 associates with p56<sup>lck</sup> upon T cell activation. Primary T cells were stimulated with anti-CD3 and CD28 antibodies for the indicated times. From cell lysates, p56<sup>lck</sup> was immunoprecipitated, and the precipitates were analyzed by immunoblotting with anti-ubiquitin (*Ubi*), anti-SOCS-6, and anti-p56<sup>lck</sup> antibodies.

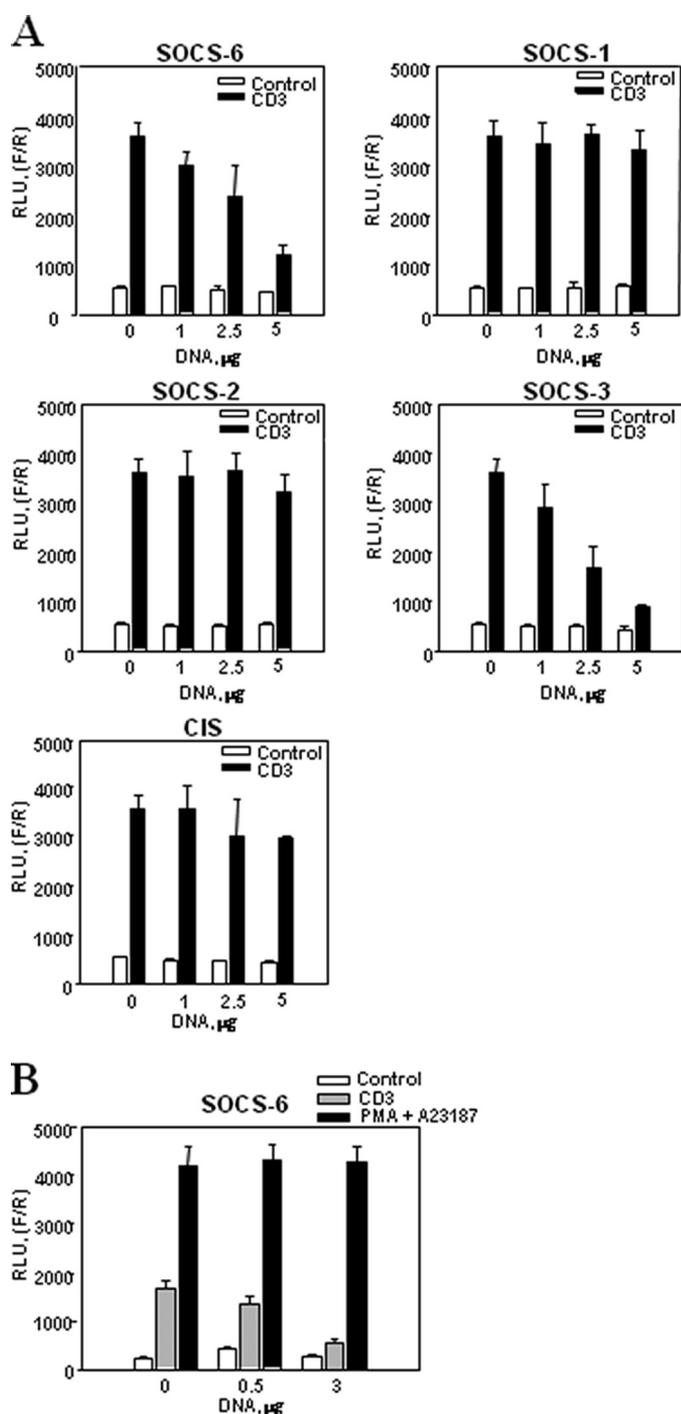
As shown in Fig. 5E, SOCS-6 expression was up-regulated 1 h after T cell activation. Second, we investigated SOCS-6-p56<sup>lck</sup> binding in primary T cells. In primary T cells stimulated by CD3 + CD28 cross-linking, SOCS-6 coimmunoprecipitates with p56<sup>lck</sup> 1 h after stimulation (Fig. 5F, middle panel). Moreover, the level of ubiquitinated-p56<sup>lck</sup> was detected starting from 1 h (Fig. 5F, upper panel), the time of which is coincident with that of SOCS-6 up-regulation and the SOCS-6-p56<sup>lck</sup> binding. These results in primary T cells further support that SOCS-6 associates with and promotes ubiquitination of p56<sup>lck</sup>.

**SOCS-6 Inhibits TCR Stimulation-dependent Activation of the IL-2 Promoter**—Having established SOCS-6-dependent regulation of p56<sup>lck</sup> levels, we asked whether SOCS-6 inhibits T cell activation. Jurkat T cells were transfected with plasmids expressing SOCS-6 along with the reporter plasmid, IL-2-Luc, and stimulated by plating them on anti-CD3 antibody-coated plates. As shown in Fig. 6A, SOCS-6 expression repressed IL-2 promoter-driven transactivation in a dose-dependent manner. SOCS-3 overexpression also resulted in the repression of the IL-2 promoter activity as reported previously (40, 41). However, SOCS-1, SOCS-2, and CIS cannot repress the IL-2 promoter (Fig. 6A) supporting the specificity of SOCS-6-mediated repression. As a control, we examined the effect of SOCS-6 on PMA plus A23187-stimulated T cell activation. As shown in Fig. 6B, SOCS-6 showed no inhibitory effect on T cell activation induced by such treatment, indicating that SOCS-6 negatively regulates TCR-proximal signaling events upstream of PMA/ionomycin. Taken together, these results establish SOCS-6 as a negative regulator of TCR stimulation-dependent T cell activation.

## DISCUSSION

Here, we describe a novel function of SOCS-6 in response to TCR

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**FIGURE 6. SOCS-6 inhibits T cell activation.** A, SOCS-6 inhibits TCR-dependent IL-2 promoter activation in a dose-dependent manner. Jurkat T cells were transfected with 5  $\mu$ g of IL-2-Luc and 10 ng of tk-R-Luc reporter along with 0, 1, 2.5, and 5  $\mu$ g of plasmids expressing SOCS-6, SOCS-1, SOCS-2, SOCS-3, and CIS. *F*, firefly; *R*, Renilla. At 24 h post-transfection, cells were stimulated by CD3 cross-linking and processed as described under "Experimental Procedures." RLU, relative luciferase units. B, SOCS-6 inhibits TCR-proximal signaling events upstream of PMA/ionomycin. Jurkat T cells were transfected and stimulated by CD3 cross-linking or treatment with PMA + A23187 as described under "Experimental Procedures." Experiments were performed three times in duplicate, and S.D. was indicated.

stimulation, SOCS-6 associates with and negatively regulates p56<sup>lck</sup>. We also show that negative regulation involves ubiquitination and proteasomal degradation of activated p56<sup>lck</sup>. SOCS-6 colocalized with active p56<sup>lck</sup> at the immunological

synapse formed upon T cell-APC conjugation. Because p56<sup>lck</sup> is essential for activation of mature T cells, these findings provide a novel regulation mode functioning in T cell activation, which is complementary to other mechanisms such as Csk kinase activity (13), CD45 dephosphorylation (14), HSP 90-mediated ubiquitination (19), and Cbl-mediated proteasomal degradation (17).

Although originally identified in SOCS family proteins, the SOCS box is seen in more than 40 proteins (42). Recent evidence shows that SOCS box-containing proteins regulate levels of specific proteins by mediating their poly-ubiquitination and subsequent degradation. In this process, the SOCS box bridges specific substrates destined for degradation and the E3 ubiquitin protein ligase complex containing elongin B and C (43). However, to date, there is no evidence supporting the idea that SOCS-6 promotes proteasomal degradation of interacting proteins such as phosphatidylinositol 3-kinase, IRS-2, or IRS-4; thus p56<sup>lck</sup> is the first protein whose levels are known to be regulated through interaction with SOCS-6. To interact with p56<sup>lck</sup>, the KID (aa 47–218) in the SOCS-6 N terminus provides an interface for the kinase domain of p56<sup>lck</sup>. Binding of KID to p56<sup>lck</sup> appears to be highly specific, because no other kinase domain of the various protein-tyrosine kinases tested showed SOCS-6 binding (Fig. 1). However, we do not exclude the possibility that the KID domain acts as a protein-protein interaction domain for kinases not tested. Compared with SOCS-1–3, SOCS-4–7 contain long N-terminal domains of ~400 amino acids (44). These N-terminal domains have been thought to function in recognition of substrate proteins to be ubiquitinated, although evidence supporting this idea is limited. Our finding that KID recognizes the ubiquitination target, p56<sup>lck</sup>, provides evidence supporting this notion.

A recent report showed that p56<sup>lck</sup> ubiquitination was enhanced when the SH2 and SH3 inhibitory interaction was released but that ubiquitination did not require p56<sup>lck</sup> kinase activity or phosphorylation of Ser-42 and Ser-59 or Tyr-394 (19), implying that p56<sup>lck</sup> is readily ubiquitinated in the open conformation, as is p56<sup>lck</sup> (F505). Consistent with this idea, our data show that SOCS-6-mediated ubiquitination and degradation are specific for the active pool of p56<sup>lck</sup>. SOCS-6 interacts with p56<sup>lck</sup> (F505) but not with p56<sup>lck</sup> (WT) (Fig. 3A), and SOCS-6-mediated degradation was observed only with p56<sup>lck</sup> (F505) (Fig. 5B). SOCS-6-p56<sup>lck</sup> binding was induced following TCR stimulation with maximal binding observed 60–120 min after stimulation in Jurkat T cells (Fig. 3B) and 60 min after stimulation in primary T cells (Fig. 5F). Consistently, as shown in Fig. 4, phosphorylated p56<sup>lck</sup> completely colocalized with SOCS-6 at the IS 60 min after stimulation, a time coinciding with maximal binding. These findings support the model that, during initial activation of TCR signaling, SOCS-6 and p56<sup>lck</sup> are differentially located, allowing the active form of p56<sup>lck</sup> to activate downstream signaling. After ~1 h, under the experimental conditions tested, SOCS-6 translocates to the IS and associates with and promotes the ubiquitination of active p56<sup>lck</sup>, thereby targeting it for proteasomal degradation.

Previously, c-Cbl was reported involved in proteasomal degradation of p56<sup>lck</sup> (16). c-Cbl participates in regulating p56<sup>lck</sup> by dislodging the kinase from the plasma membrane lipid raft and

then targeting it to the E3 ubiquitin ligase complex, resulting in proteasomal degradation of p56<sup>lck</sup> (16, 17). Indeed, a c-Cbl-deficient T cell line showed marked deficiency in p56<sup>lck</sup> ubiquitination and increased levels of active p56<sup>lck</sup>. However, in a c-Cbl-deficient cell line, ubiquitinated p56<sup>lck</sup> was still observed in the presence of a proteasome inhibitor (17), implying that c-Cbl is not the only mediator of p56<sup>lck</sup> degradation. It will be of interest to examine levels of ubiquitinated p56<sup>lck</sup> in cells deficient in both SOCS-6 and c-Cbl. In addition to c-Cbl, several E3 ubiquitin ligases, such as Cbl-b, Grail, Itch, and Nedd4, are involved in inducing and maintaining immune self-tolerance (45). These proteins induce T cell anergy by targeting components of TCR signaling for proteasomal degradation as follows: for example, c-Cbl targets p56<sup>lck</sup>, ZAP-70, and CD3- $\zeta$ , and Cbl-b targets phospholipase C- $\gamma$  and protein kinase C $\theta$  (45). These previous findings raise the possibility that SOCS-6 functions to induce and maintain anergy. Our biochemical data show that ectopic expression of SOCS-6 inhibits T cell activation by promoting proteasomal degradation of active p56<sup>lck</sup>, implying that lack of SOCS-6 might lead to accumulation of active p56<sup>lck</sup> upon TCR stimulation and thereby result in abnormal development or hyper-responsiveness of T cells. However, the number of lymphocytes was reported to be normal in mice lacking SOCS-6 (31). This phenomenon could be explained by functional complementation by other SOCS proteins and/or c-Cbl. For example, overexpression of SOCS-3 inhibits IL-2 production in TCR-mediated T cell activation (Fig. 6) (40, 41) by inhibiting calcineurin-dependent dephosphorylation of NF-ATp or presumably by interacting with p56<sup>lck</sup> (25, 40).

T cells from systemic lupus erythematosus patients exhibit underlying defects in TCR-mediated proximal signaling events. Moreover, p56<sup>lck</sup> deficiency has been reported in T cells of patients suffering from diseases such as systemic lupus erythematosus and type I diabetes (46–48). Reduced p56<sup>lck</sup> expression parallels increased ubiquitination. Still, little is known about the mechanisms underlying increased p56<sup>lck</sup> ubiquitination in T cells from systemic lupus erythematosus patients, although Cbl and CD45 may contribute to its down-regulation. It will be interesting to examine expression or function of SOCS-6 in T cells from systemic lupus erythematosus or type I diabetes patients.

In conclusion, we have identified SOCS-6 as a p56<sup>lck</sup>-associated protein targeting p56<sup>lck</sup> to proteasome-dependent degradation in response to T cell activation. Negative regulation of p56<sup>lck</sup> by SOCS-6 may in part prevent uncontrolled T cell activation.

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**SOCS-6 Negatively Regulates T Cell Activation through Targeting p56<sup>lck</sup> to Proteasomal Degradation**

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