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Src-like Adaptor Protein (SLAP) Is a Negative Regulator of T Cell Receptor Signaling

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Abstract

Initiation of T cell antigen receptor (TCR) signaling is dependent on Lck, a Src family kinase. The Src-like adaptor protein (SLAP) contains Src homology (SH)3 and SH2 domains, which are highly homologous to those of Lck and other Src family members. Because of the structural similarity between Lck and SLAP, we studied its potential role in TCR signaling. Here, we show that SLAP is expressed in T cells, and that when expressed in Jurkat T cells it can specifically inhibit TCR signaling leading to nuclear factor of activated T cells (NFAT)-, activator protein 1 (AP-1)-, and interleukin 2-dependent transcription. The SH3 and SH2 domains of SLAP are required for maximal attenuation of TCR signaling. This inhibitory activity can be bypassed by the combination of phorbol myristate acetate (PMA) and ionomycin, suggesting that SLAP acts proximally in the TCR signaling pathway. SLAP colocalizes with endosomes in Jurkat and in HeLa cells, and is insoluble in mild detergents. In stimulated Jurkat cells, SLAP associates with a molecular signaling complex containing CD3 ζ , ZAP-70, SH2 domain-containing leukocyte protein of 76 kD (SLP-76), Vav, and possibly linker for activation of T cells (LAT). These results suggest that SLAP is a negative regulator of TCR signaling.

Key words: Lck • Src homology 2 • T cell activation • calcium flux • endosomes

Introduction

Activation of the TCR is a critical event for T cell development in the thymus, as well as for initiating immune responses in mature T cells. The TCR is devoid of intrinsic catalytic activity, yet its engagement leads to significant induction of protein tyrosine kinase activity, resulting in phosphorylation of tyrosine residues on many proteins. Src family protein tyrosine kinases, especially in T cells of one of its members, Lck, have been shown to be responsible for initiating this process by phosphorylating tyrosines within immunoreceptor tyrosine-based activation motifs (ITAMs)¹

present within CD3 and ζ chains of the TCR (1). Subsequently, tyrosine-phosphorylated ITAMs serve as docking sites for Src homology (SH)2 domains present in the two members of another family of tyrosine kinases, ZAP-70 and Syk. Recruitment of ZAP-70/Syk to the TCR complex activates these kinases which, in concert with Lck, transduce the TCR signal downstream by phosphorylating several targets. This activation process ultimately leads to the transcription of genes such as IL-2, a hallmark of T cell activation.

The essential role of Lck in TCR signaling has been underscored by the demonstration that a Jurkat T cell line lacking expression of this kinase is unresponsive to TCR stimulation (2). Moreover, mice deficient in or expressing a dominant negative form of Lck display a profound blockade in thymocyte development in the transition to the CD4/CD8 double-positive stage (3, 4). ITAM phosphorylation is abrogated in both the Lck-deficient Jurkat line and Lck-deficient thymocytes (5, 6).

Lck, like other Src family members, has a distinctive structure. It has a unique NH₂-terminal region, followed by SH3, SH2, catalytic (SH1), and COOH-terminal regulatory do-

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¹Abbreviations used in this paper: AP-1, activator protein 1; CI-M6PR, cation-independent mannose 6-phosphate receptor; FLAG, fluoresceinated antigen; GST, glutathione S-transferase; HRP, horseradish peroxidase; IP, immunoprecipitation; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T cells; NFAT, nuclear factor of activated T cells; PDGFR, platelet-derived growth factor receptor; PTyr, phosphotyrosine; SH, Src homology; SLAP, Src-like adaptor protein; SLP-76, SH2 domain-containing leukocyte protein of 76 kD; TAG, large T antigen; WCL, whole cell lysate; WT, wild-type.

mains. Two functions have been attributed to the unique region of Lck: (a) myristoylation and palmitoylation of this region target Lck to the plasma membrane, and (b) a cysteine-containing motif enables it to associate with CD4 or CD8 coreceptors (7–10). The SH3 and SH2 domains are well-defined protein–protein interaction modules (11) and, as such, facilitate binding of Lck to many signaling molecules. The SH3 domain can recognize proline-rich sequences within c-Cbl, the p85 subunit of phosphatidylinositol 3-kinase, Ras–GTPase-activating protein, hematopoietic specific protein 1, and CD2 (12–16). The SH2 domain can interact with tyrosine-phosphorylated ZAP-70 and Syk kinases (17, 18) or with the negative regulatory site of Lck in an intramolecular interaction (19). Following the SH2 domain is the catalytic domain of Lck. Activation of the kinase is dependent on autophosphorylation of a single tyrosine residue within the activation loop of the kinase domain. Dephosphorylation of a well-conserved tyrosine present in the COOH-terminal regulatory region is also required for kinase activation (for a review, see reference 20).

A molecule structurally resembling Src family protein tyrosine kinases was cloned using a yeast two-hybrid screen (21). Like Lck, this Src-like adaptor protein (SLAP) contains a short unique NH₂-terminal domain, followed by SH3 and SH2 domains. However, it does not contain a tyrosine kinase domain. Both the SH3 and SH2 domains of SLAP are highly homologous to those of Lck and share with them 55 and 50% identity, compared with 42 and 43% with c-Src, respectively (K best nonintersecting alignments; ExPASy, available at <http://www.expasy.ch>). The SLAP SH3 and SH2 domains share less homology with other SH3 domains (those of phospholipase C- γ 1 and the p85 α subunit of phosphatidylinositol 3-kinase share only 20 and 24% identity, respectively) and SH2 domains (those of Shc and Grb2 share only 25 and 27% identity, respectively [21]). Instead of a kinase domain, SLAP contains a unique COOH terminus of 104 residues. The initial study where SLAP was identified suggested ubiquitous expression of SLAP mRNA among tissues tested. In a subsequent study, microinjected SLAP inhibited a platelet-derived growth factor receptor (PDGFR)-dependent mitogenic response in fibroblasts (22). Biochemical analysis revealed that SLAP associated with activated PDGFRs, and that it could efficiently compete with Src for PDGFR binding.

Since the original report suggested the possibility of its expression in T cells, we studied the potential role of SLAP in regulating TCR signaling. Here, we report that SLAP is expressed in T lineage cells, and that it can inhibit TCR signaling when expressed in the Jurkat T cell line.

Materials and Methods

Cell Lines and Reagents. Jurkat cells or Jurkat cells transfected with the simian virus 40 large T antigen (SV40 TAG; provided by G. Crabtree, Stanford University, Stanford, CA) and murine cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, penicillin, streptomycin, and glutamine. HeLa cells were passaged in DME supplemented with 5% FCS. The Ab used

for TCR stimulation of Jurkat TAG cells was C305, an anti-Ti β chain mouse mAb (23). Blotting Abs were as follows: an anti-fluoresceinated antigen (FLAG) M2 mAb (Sigma Chemical Co.); an antiphosphotyrosine (PTyr) mAb, 4G10 (Upstate Biotechnology); an anti-CD3 ζ mAb, 6B10.2 (24); rabbit anti-linker for activation of T cells (LAT) serum (25); sheep anti-SLP-76 serum (26); anti-ZAP-70 mAb, 2F3.2 (5); rabbit anti-Vav serum (Santa Cruz Biotechnology); rabbit anti- α -tubulin serum (Sigma Chemical Co.); and anti-Lck mAb, 1F6 (provided by Dr. J. Bolen, Bristol-Meyers Squibb, Wallingford, CT). Rabbit serum (C1661) directed against the NH₂-terminal amino acids (6–25) of murine SLAP was generated in our laboratory. Two secondary reagents were used: a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Southern Biotechnology Associates), and HRP-conjugated protein A (Amersham). For immunofluorescence studies, we used rabbit anti-murine SLAP C1661 polyclonal Ab or the M2 anti-FLAG mAb to detect FLAG-tagged murine SLAP, or rabbit anti-cation-independent mannose 6-phosphate receptor (CI-M6PR) polyclonal antiserum (gift of F. Brodsky, University of California at San Francisco, San Francisco, CA) to detect CI-M6PR. FITC-conjugated goat anti-rabbit IgG (GIBCO BRL) or Texas red-conjugated goat anti-mouse IgG (Caltag) was used as secondary Ab.

DNA Constructs. All expression constructs used to express SLAP protein contained murine SLAP cDNA fused to the FLAG epitope tag at its COOH terminus. SLAP cDNA–FLAG inserts were flanked by BamHI–XhoI sites, and were subcloned into BamHI–SalI sites of pEF-BOS X-C, a mammalian expression vector (27). The pEF-SLAP SH3* had a CCA to CTC mutation, changing a P to L at position 73; the pEF-SLAP SH2* had a CGA to GAA mutation, changing an R to E at position 144; pEF-SLAP Δ C expressed a truncated version of SLAP, including the NH₂ terminus, the SH3 and SH2 domains, and 10 amino acids of the unique COOH terminus (i.e., 1–187). Glutathione S-transferase (GST)–SLAP, GST–Lck(SH2), and pEF-TacT were constructed as described (18, 21, 28). The nuclear factor of activated T cells (NFAT) luciferase and IL-2 luciferase reporters were provided by G. Crabtree. Activator protein 1 (AP-1) luciferase was described previously (29). pRc/ β gal was a gift from M. Karin (University of California, San Diego, CA).

Northern Blot Analysis. Tissues were derived from C57BL/6 mice. Total RNA was extracted using RNazol (Tel-Test). 10 μ g of total RNA per sample was stained with ethidium bromide, resolved on agarose gel, and transferred onto MagnaCharge Nylon membrane (Micron Separations, Inc.). The blot was hybridized with a [³²P]dCTP-labeled XcmI–ScaI fragment encompassing 330 nucleotides encoding the unique COOH terminus of murine SLAP.

Transfections and Luciferase Assays. 2×10^7 Jurkat TAG cells were resuspended in 400 μ l of serum-free RPMI medium containing plasmid DNA, placed in a 0.4-cm cuvette, and electroporated at 250 V, 960 μ F using the Bio-Rad Gene Pulser electroporator. Cells were harvested after 16–24 h, and were stimulated with soluble anti-TCR Ab C305 (1:1,000 dilution of ascites), PHA (1 μ g/ml), PMA (50 ng/ml), or ionomycin (1 μ M), or with the indicated combination of reagents. After 8 h, cells were lysed and assayed for luciferase activity as described (30). All transfections included the pRc/ β gal plasmid (31) to normalize for differences in gene transfer by assay of β -galactosidase activity, which was performed using a chemiluminescence assay system (Tropix).

Cell Enrichment and Immunoprecipitations. Jurkat TAG cells were transiently cotransfected with pEF-TacT and the indicated effector plasmids. After 12 h, cells were harvested, washed in PBS, and resuspended in MACS[®] purification buffer (PBS, 0.5% BSA, 2 mM EDTA; Miltenyi Biotec). Cells were then stained with FITC-

conjugated anti-Tac mAb (anti-CD25 FITC; Becton Dickinson) followed by anti-FITC MultiSort MicroBeads (Miltenyi Biotec), and were purified on a VS⁺ column according to the manufacturer's protocol. The purity of isolated cells was determined using flow cytometry (FACScanTM; Becton Dickinson). Tac⁺ enriched populations were lysed in lysis buffer containing 1% NP-40, 10 mM Tris (pH 7.8), 150 mM NaCl, 2 mM EDTA, and protease and phosphatase inhibitors. Lysates were normalized for protein concentration using the Bradford assay (Bio-Rad). After addition of SDS to a final concentration of 1%, lysates were incubated on ice for 10 min, boiled for 5 min, diluted to final 0.5% SDS with 1% NP-40 lysis buffer, briefly sonicated to shear the genomic DNA, and centrifuged at 100,000 *g* for 30 min. Soluble lysates were incubated with 1 μ g of rabbit anti-murine SLAP C1661 polyclonal Ab cross-linked to protein A-Sepharose beads (Amersham Pharmacia Biotech) for 2 h. Beads were washed four times with 1% NP-40 buffer, and were boiled for 5 min in SDS sample buffer. A standard protocol for Western blotting was followed: proteins were separated by SDS-PAGE, transferred to Immobilon-P (Millipore) membrane, and immunoblotted with C1661 Ab followed by a protein A conjugated to HRP (Amersham). Enhanced chemiluminescence was used to detect immunoreactive proteins (Amersham).

Measurement of Intracellular Calcium. Tac⁺ cells were selected as described above, and were cultured for 2 h to allow cell recovery from the purification procedure. To assay intracellular calcium concentration, cells were loaded with Indo-1 (Molecular Probes), washed, and then stimulated with C305 mAb (1:1,000) followed by ionomycin (1 μ M). The fluorescence intensities at 400 and 500 nm wavelengths were measured using a Hitachi F-4500 spectrofluorometer, and were used to calculate concentration of intracellular calcium (32).

Immunofluorescence Microscopy. Jurkat TAg cells were transiently transfected as above, incubated for 12–16 h, and seeded onto poly-L-lysine-coated slides. HeLa cells were transiently transfected using the calcium phosphate method, and were incubated on a chambered coverglass (Lab-Tek) to allow for cell adhesion. Next, Jurkat TAg or HeLa cells were fixed in 3.4% paraformaldehyde at room temperature for 20 min, followed by a 4-min permeabilization in 0.1% Triton X-100 in PBS, and 10 min of blocking in 0.2% BSA in PBS. Cells were incubated with primary Abs for 30 min, washed twice with 0.2% BSA in PBS, and stained with secondary Abs. MOWIOL[®] (Calbiochem) supplemented with 2.5% DabcoTM (Sigma Chemical Co.) was used to mount the coverslips and prevent photobleaching. Confocal fluorescence microscopy was performed using a Bio-Rad MRC1000 confocal imaging system interfaced to a Zeiss inverted microscope equipped with a 100 \times NA 1.3 objective.

GST Pulldowns, Immunoprecipitations, and Western Blotting. Jurkat cells were left untreated or were stimulated with C305 mAb (1:1,000 dilution of ascites) for 2 min, and lysed in 1% NP-40 lysis buffer. Procedures for GST pulldowns and immunoprecipita-

tions (IPs) were described previously (18). For GST pulldown experiments, 2 μ g of each GST fusion protein was incubated with lysates obtained from 5×10^7 cells for 2 h. For IPs, 1 μ g of rabbit anti-SLAP C1661 affinity-purified Ab or 1 μ g of anti-Lck 1F6 mAb was incubated with lysates obtained from 3×10^7 transfected cells. Both Abs were cross-linked to protein A-Sepharose beads (Amersham Pharmacia Biotech) before IPs. A standard protocol for Western blotting was followed (see above).

Results

Tissue- and Cell Type-specific Expression of SLAP mRNA.

As the first step in our analysis of SLAP, we studied its tissue-specific expression in mouse tissues and cell lines. SLAP mRNA is predominantly expressed in lymphoid tissues, though low levels can be detected in the lung and brain (Fig. 1 A). To further elucidate the cell types that express SLAP mRNA, we compared message levels in purified splenic T cells, purified splenic B cells, and in various cell lines representing fibroblasts, macrophages, mast cells, B cells, and T cells (Fig. 1 B). Note that EL-4 thymoma was the only murine cell line we analyzed that expressed significant levels of the message. After longer exposure of the Northern blot, Raw264.7 and P815 cells had detectable levels, but NIH 3T3 cells did not. Strikingly, both primary T cells and primary B cells expressed significant levels of SLAP mRNA. Based on these data, we conclude that T lineage cells and B lineage cells are the predominant cell types expressing SLAP.

Effect of SLAP Expression on TCR-induced Transcriptional Responses. Having demonstrated endogenous SLAP transcripts in thymocytes and primary T cells, we wanted to study the functional consequences of the presence of SLAP protein in T cells. We analyzed the effects of SLAP expression in Jurkat TAg cells on one measure of TCR signaling, the transcriptional activity of the IL-2 promoter and its two elements, NFAT and AP-1. Although Jurkat and Jurkat TAg cells expressed SLAP mRNA, we have not been able to detect SLAP protein in these cell lines (data not shown).

We started our analysis by using a very sensitive reporter of TCR signaling, the luciferase gene under the control of an NFAT element arranged in triplicate (NFAT luciferase). Transient expression of SLAP in Jurkat TAg cells potentially inhibited TCR-driven NFAT transcription in a dose-dependent manner (Fig. 2 A). To determine whether the inhibitory effects of SLAP can occur at physiological amounts of the protein, we compared the levels of SLAP in these transfected Jurkat TAg cells (cells were from the same ex-

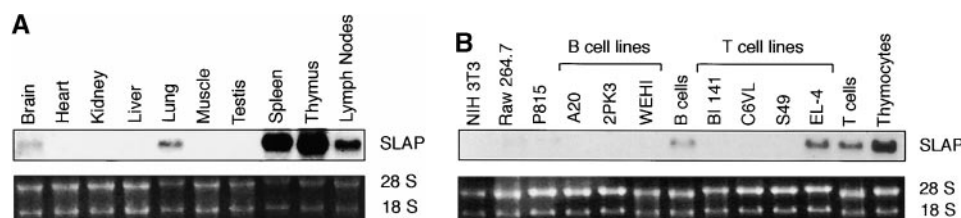


Figure 1. SLAP is predominantly expressed in the lymphoid tissues. Northern blot analysis was performed on (A) multiple tissues, (B) various cell lines (fibroblast [NIH 3T3], macrophage [Raw264.7], mastocytoma [P815], B cell lines, and T cell lines), and enriched splenic B and T cells (>92% and 98% pure, respectively). A ³²P-labeled DNA fragment

from the unique COOH terminus of SLAP cDNA was used to detect SLAP mRNA present in 10 μ g of total RNA. Equal loading and complete transfer of RNA were confirmed by ethidium bromide staining and quantitation of ribosomal 28S and 18S RNA using the Bio-Rad Gel Doc 1000 system (bottom panels).

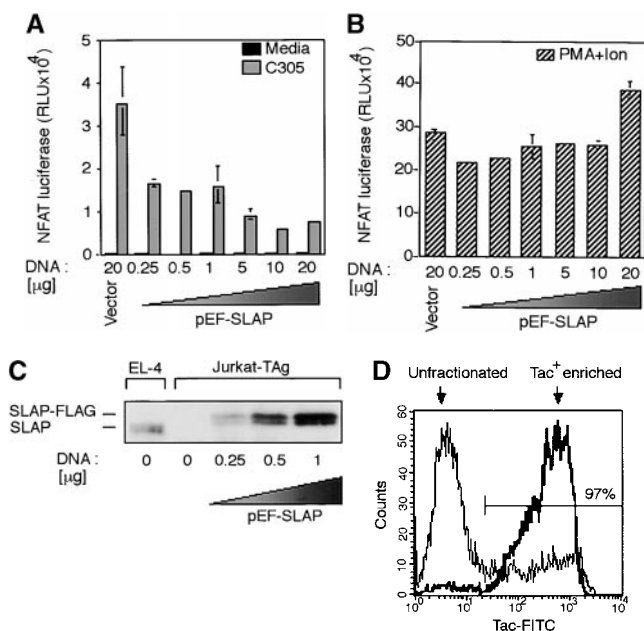


Figure 2. SLAP inhibits TCR induction of NFAT-driven transcription in a dose-dependent manner. Jurkat TAG cells were cotransfected with four different plasmids: a luciferase reporter under the transcriptional control of a multimerized NFAT element (NFAT luciferase); a transfection efficiency indicator (pRc/ β gal); a transfection tag (pEF-TacT); and an effector plasmid (either pEF-SLAP or an empty vector, pEF-BOS). The total amount of DNA was kept constant by supplementing the various amounts of pEF-SLAP plasmid with an empty vector. After 16–24 h, cells from each transfection were tested for the transfection efficiency by β -galactosidase assay (data not shown), or incubated for an additional 8 h with medium alone (Media), soluble anti-TCR mAb (C305), or PMA and ionomycin (PMA+Ion), and were assayed for luciferase activity. Luciferase activity was normalized to β -galactosidase and is presented in relative light units (RLU; A and B). Cells were also tested for the level of SLAP protein: transfections containing 0.25, 0.5, or 1.0 μ g of pEF-SLAP were enriched for Tac⁺ cells by magnetic beads (D) and used for IPs of the ectopically expressed SLAP (C; see Materials and Methods). As positive and negative controls for the presence of murine SLAP, EL-4 and nontransfected Jurkat TAG cells were used, respectively.

periment shown in Fig. 2, A and B) with the level present in a murine thymoma line EL-4. To ensure appropriate comparison of EL-4 to only those Jurkat TAG cells that were successfully transfected with SLAP, we also had cotransfected a tag to mark the transfected cells—a plasmid encoding the extracellular and transmembrane domains of Tac (IL-2R α chain). By enriching for Tac-expressing cells using magnetic beads, we separated SLAP-expressing cells from the nontransfected cells (populations were >97% Tac⁺; e.g., the purification profile for cells transfected with 0.25 μ g of SLAP is shown in Fig. 2 D). We used this purification approach since we and others have established that Jurkat cells proportionately coexpress cotransfected plasmids (30, 33). By immunoprecipitating SLAP from equivalent amounts of cell lysates, we could appropriately compare the level of SLAP in the transfected population to the level present in EL-4. We established that 0.25–0.5 μ g of transfected pEF-SLAP plasmid resulted in levels of SLAP protein comparable with that present in EL-4 cells (Fig. 2 C). Significantly, even the transfection of 0.25 μ g of pEF-

SLAP plasmid significantly inhibited the NFAT reporter. These results indicate that SLAP can negatively regulate TCR signaling when expressed at approximately physiological (EL-4 cells) levels (Fig. 2, A and C).

This suppressive effect was not limited to the NFAT element, since SLAP could also inhibit TCR-dependent activation of AP-1 and IL-2 luciferase reporters (Fig. 3, A and C). To stimulate the IL-2 luciferase reporter, we used a combination of PHA and PMA, an effective stimulus known to induce transcriptional activity of the IL-2 promoter in a TCR-dependent manner (34). SLAP did not interfere with the general transcriptional machinery in a non-specific manner, since it had no effect on NFAT, AP-1, or the IL-2 promoter in cells stimulated with PMA and the calcium ionophore (ionomycin; Fig. 2 B and Fig. 3, B and D), nor did it suppress the luciferase activity under the control of the constitutive Rous sarcoma virus (RSV) promoter (data not shown). The ability of PMA and ionomycin—pharmacological agents that bypass proximal TCR signaling by activating Ras and increasing intracellular calcium, respectively—to activate transcription in the presence of SLAP places the SLAP-imposed blockade more proximal in the TCR signaling pathway than Ras activation and calcium increases. Moreover, TCR-dependent activation

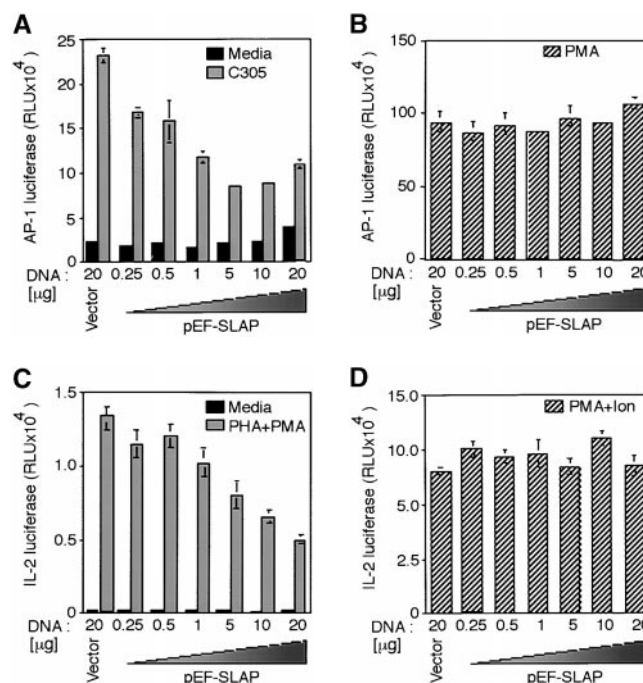


Figure 3. SLAP inhibits TCR induction of both AP-1- and IL-2 promoter-driven transcription in a dose-dependent manner. Cells were cotransfected with three different plasmids: a luciferase reporter under the transcriptional control of either AP-1 element (A and B) or IL-2 promoter (C and D); a transfection efficiency indicator (pRc/ β gal); and an effector plasmid (either pEF-SLAP or an empty vector, pEF-BOS). After 16–24 h, transfected cells were assayed for β -galactosidase activity, and stimulated with either C305 (A), PMA (B), PHA and PMA (C), or a combination of PMA and ionomycin (PMA+Ion, D), as indicated (see Materials and Methods). The activity of each reporter is presented as described in the legend to Fig. 2.

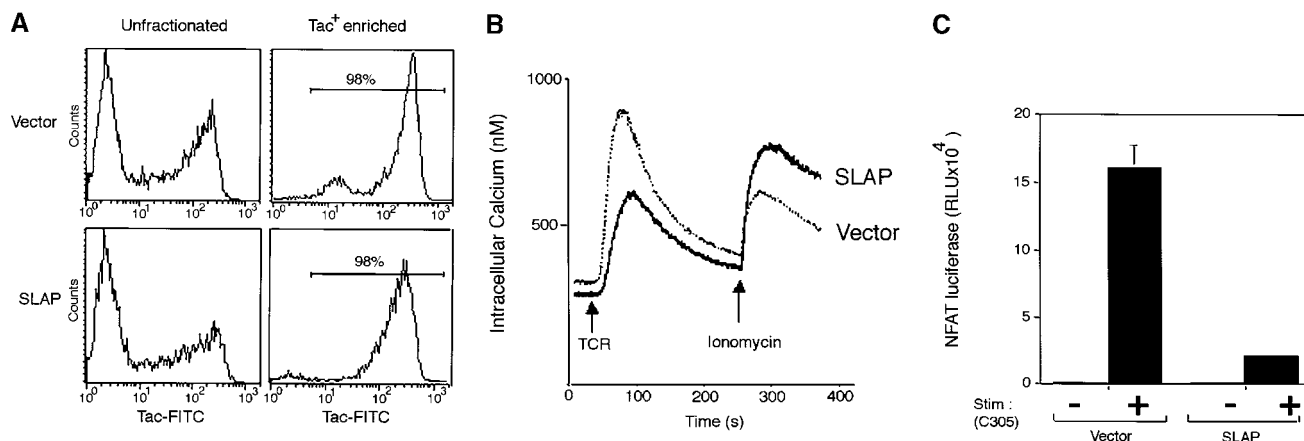


Figure 4. SLAP inhibits TCR-dependent Ca^{2+} mobilization in Jurkat cells. Jurkat TAG cells were transiently cotransfected with three plasmids: NFAT luciferase, pEF-TacT, and pEF-SLAP or an empty vector. 16 h later, transfected cells were enriched based on expression of Tac on the cell surface (see Materials and Methods). Highly enriched populations (A) were loaded with the fluorescent dye indicator Indo-1, then stimulated with soluble anti-TCR mAb (C305) followed by ionomycin (arrows indicate the time of stimulation), and the change in intracellular Ca^{2+} concentration was measured as a function of time (B). Cells from the same experiment were also tested for TCR-dependent NFAT induction (C).

of both the Ras and Ca^{2+} pathways is inhibited by SLAP, since stimulation of cells with anti-TCR Ab and either PMA alone or ionomycin alone did not bypass the blockade (data not shown).

Functional Effect of SLAP Expression on TCR-induced Ca^{2+} Flux and Protein Tyrosine Phosphorylation. Since PMA and ionomycin treatment bypassed SLAP-dependent inhibition of NFAT induction, but anti-TCR plus PMA stimulation did not, we reasoned that SLAP might suppress the TCR-dependent Ca^{2+} flux. To examine this, we cotransfected SLAP or an empty vector with a plasmid encoding Tac, and enriched for Tac-expressing cells as described above (populations were >98% Tac⁺; Fig. 4 A). As shown in Fig. 4 B, cells transfected with SLAP, when compared with the vector-transfected cells, showed a substantially reduced calcium flux in response to anti-TCR stimulation. Importantly, this pattern of responsiveness was reversed upon subsequent stimulation of cells with ionomycin. Note that SLAP-transfected cells responded to the calcium ionophore by mobilizing more intracellular Ca^{2+} than vector-transfected cells. As a control in this same experiment, we also transfected the NFAT luciferase reporter plasmid to show that SLAP was expressed at levels sufficient to inhibit the TCR signaling pathway leading to NFAT activation (Fig. 4 C). We employed the same approach of cotransfection and enrichment for Tac⁺ cells to study the effect of SLAP on TCR-dependent increase in protein tyrosine phosphorylation. However, we failed to observe any differences in the pattern of tyrosine phosphorylation in total cell lysates from TCR-stimulated cells when SLAP was expressed (data not shown).

Contribution of the SH2, SH3, and the Unique COOH-terminal Domains to the Inhibitory Function of SLAP. To study the mechanism by which SLAP inhibits TCR signaling, we studied the role of the SH2, SH3, and the unique COOH terminus of SLAP. As shown in Fig. 5, inactivation of the

SLAP SH2 domain by replacing an arginine with a glutamic acid in the "FXXR" motif in the phosphotyrosine binding pocket had a profound effect on the SLAP inhibitory activity. The SLAP SH2 mutant displayed only a weak inhibi-

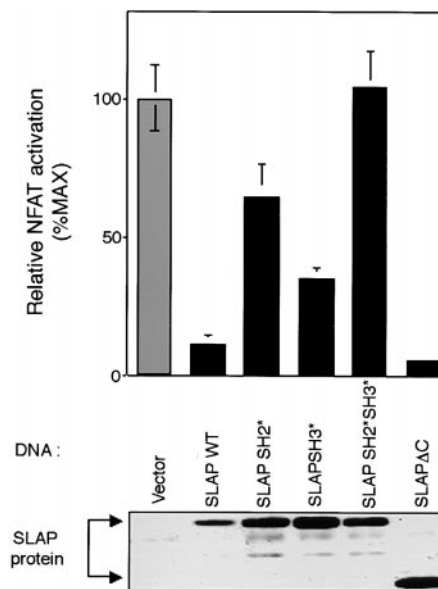


Figure 5. Both SH2 and SH3 domains of SLAP participate in inhibition of anti-TCR-induced NFAT activity. Jurkat TAG cells were cotransfected with an NFAT luciferase reporter and various amounts of one of the following effector plasmids: a WT SLAP (WT), an SH2 domain point mutant (SH2*), an SH3 domain point mutant (SH3*), an SH2 and SH3 domain double point mutant (SH2*SH3*), and a mutant with deleted COOH terminus (ΔC). An empty vector was used as a control, and the total amount of DNA was kept constant by adding empty vector DNA. Individual transfections were matched based on the expression level of the various SLAP proteins (bottom). TCR-dependent increase in NFAT activity was calculated as a fold induction, and represents a ratio of luciferase counts of anti-TCR over media-treated cells. Fold induction obtained for vector-transfected cells was defined as maximum (100% MAX).

tory effect, despite being expressed at higher levels than the wild-type (WT) protein. Inactivation of the SH3 domain by replacing a conserved proline with leucine at position 73 (35) had a more modest effect on SLAP inhibitory function. However, when both the SH2 and the SH3 domains were mutated in the same construct, no inhibition of TCR signaling was observed, suggesting that both domains contribute to the SLAP inhibitory function in an additive manner. In contrast, the unique COOH terminus of SLAP did not play any role in the inhibition, since a mutant lacking this domain (SLAP Δ C) suppressed NFAT induction to the same extent as the WT protein.

Subcellular Distribution of SLAP. We extended the structure–function analysis of SLAP to include subcellular localization of the WT protein and the various mutants. Indirect immunofluorescence staining showed perinuclear localization of SLAP in transiently transfected Jurkat TAG cells (Fig. 6 B). This staining was similar to that of an intracellular pool of Lck in Jurkat cells, which was reported to colocalize with the endosomal marker CI-M6PR (36). However, because Jurkat cells are small and have large nuclei and a thin rim of cytoplasm, thereby preventing good resolution of cellular structures, we used HeLa cells to aid in a more precise SLAP cellular localization. Using indirect immunofluorescence to identify CI-M6PR (Fig. 6 D, green) and SLAP (Fig. 6 E, red), then overlaying the images, the appearance of yellow confirms that the two molecules

colocalize (Fig. 6 F). Note that of two cells present in the field (Fig. 6 D), only one was transfected and expressed SLAP (Fig. 6 E). The same pattern of staining was obtained with two different primary Abs recognizing distinct epitopes on transfected SLAP (data not shown; see Materials and Methods). The observed SLAP staining was specific, since vector transfected cells did not stain with either Ab (data not shown).

Next, we asked which of the domains of SLAP played a role in its colocalization with endosomes by transfecting various mutants into Jurkat TAG or HeLa cells and staining for SLAP and CI-M6PR. Mutants with the inactivated SH2 domain (SH2* and SH2*SH3*) were the only ones that did not correctly colocalize with endosomes. Instead, they displayed diffuse vesicular staining (Fig. 6, G–I, and data not shown). This difference in staining indicates that the endosomal localization of WT SLAP was not an artifact of overexpression, since both WT and SH2 mutants were expressed at similar levels. The same pattern of staining was obtained for Jurkat cells (data not shown). Collectively, these data suggest that the SH2 domain of SLAP targets it to the cytoplasmic face of endosomes, most likely by tyrosine phosphoprotein(s).

Insolubility of SLAP in Mild Detergents. During the course of our studies, we discovered that SLAP protein is insoluble in mild detergents (Fig. 7). The insolubility of SLAP does not depend on the actin cytoskeleton or on interactions

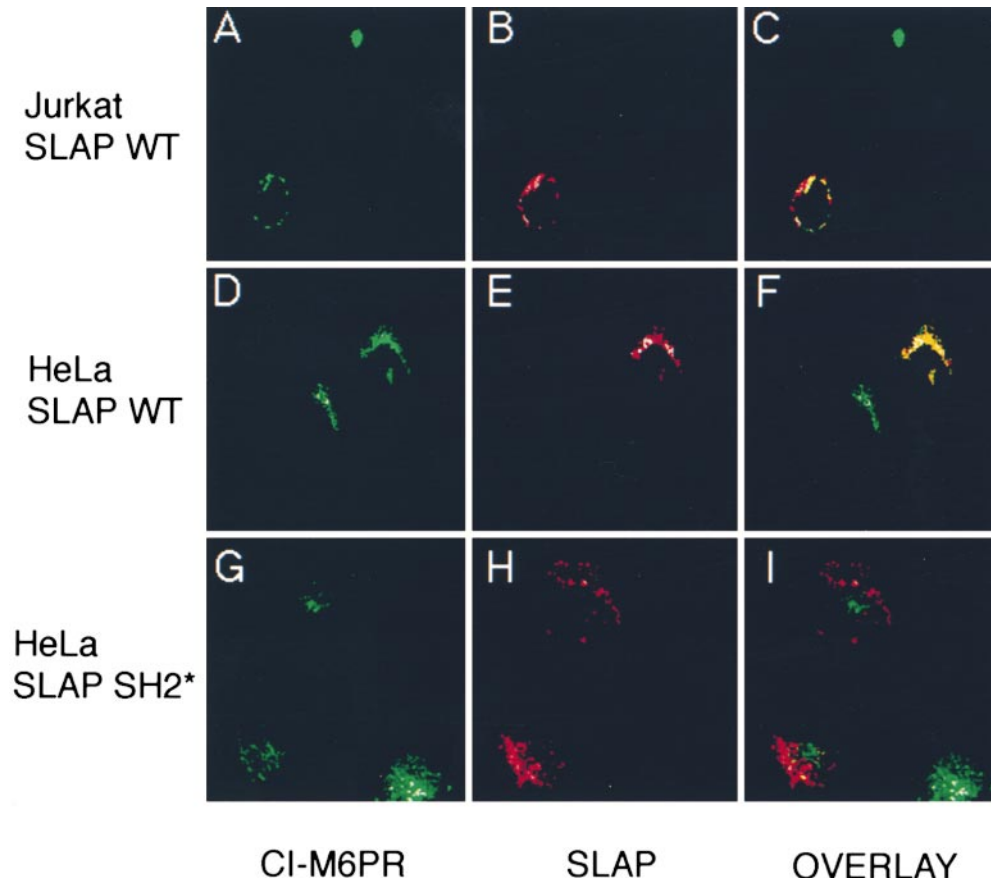


Figure 6. The SLAP SH2 domain is required for its colocalization with endosomes. Jurkat (A, B, and C) and HeLa cells (D–I) were transiently transfected with pEF-SLAP WT plasmid (A–F) or with pEF-SLAP SH2* mutant (G–I), and the indirect immunofluorescence analysis was performed (see Materials and Methods). The endogenous endosomal marker, CI-M6PR, is present in all cells (green); however, SLAP is present only in transiently transfected cells (red). To depict the degree of colocalization of SLAP and CI-M6PR, the red and green channels were merged (C, F, and I, OVERLAY). Each image represents a single section through a field of cells.

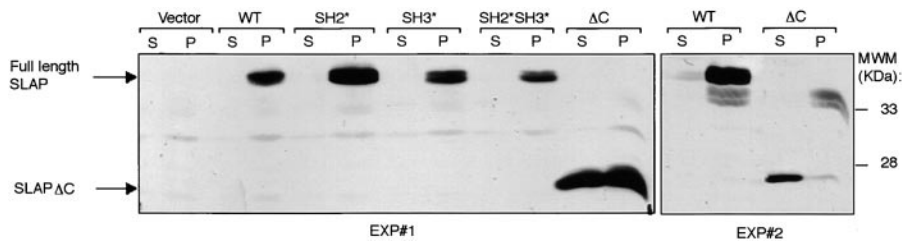


Figure 7. The unique COOH terminus of SLAP determines its insolubility in mild detergents. Jurkat cells were transiently transfected with an expression plasmid encoding either WT SLAP or the indicated mutants. After 24 h, cells were lysed in 1% NP-40 lysis buffer, and the detergent-soluble (S) and the pellet (P) fractions were separated by standard centrifugation procedure (see Materials and Methods). Pellet fractions were solubilized in the SDS-PAGE loading buffer, and cell-equivalent amounts were run on SDS-PAGE. SLAP protein was detected by Western blotting using rabbit anti-SLAP C1661 Ab. EXP, experiment; MWM, molecular weight marker.

with microtubules, since its insolubility was not sensitive to cytochalasin D or nocodazole (data not shown). This property is dependent on the unique COOH-terminal domain, since the mutant lacking this domain partitioned to the 1% NP-40 soluble fraction. The equal distribution of SLAP Δ C into soluble and pellet fractions in Fig. 7 (experiment 1) was due to high levels of expression, since the same mutant was present predominantly in the soluble fraction when expressed at lower levels in experiment 2. SLAP insolubility does not correlate with its inhibitory function because, as shown in Fig. 4, SLAP Δ C was as potent in inhibiting NFAT induction as the WT SLAP.

A Specific Subset of Phosphoproteins Interacts with SLAP. Given the critical importance of the SH2 domain for SLAP function, we wished to identify the tyrosine-phosphorylated proteins that interact with SLAP. Since WT SLAP is insoluble in the mild detergents that are used for solubilization and are necessary to preserve protein-protein interactions, we were unable to perform direct IPs of WT SLAP protein. Instead, we used two different approaches to identify the phosphoproteins that interact with SLAP. First, we used a GST pulldown experiment to detect *in vitro* interactions. Among many inducibly tyrosine-phosphorylated proteins present in Jurkat lysates, 24-kD phosphoproteins (pp24), pp36/38, pp76, and pp95 were consistently bound by GST-SLAP (Fig. 8, see arrowheads and bracket). Other bands present in Fig. 8, notably the bands corresponding to putative pp34, pp46, and pp60, were not consistently seen. GST did not retain any substantial amounts of phosphoproteins by itself, indicating the specificity of the interactions. Notice that an almost identical set of proteins was retained by the GST-Lck SH2 domain, but not by the GST-Grb2 construct. These results suggest that a subset of TCR-induced tyrosine phosphoproteins interact specifically with SLAP *in vitro*.

Our second approach was aimed at identification of *in vivo* interactions, and took advantage of the solubility of the SLAP Δ C mutant. We reasoned that this mutant should bind to the same target phosphoproteins as WT SLAP, since both proteins similarly inhibited TCR-induced NFAT activation, and they both localized to the same cellular structures. We compared anti-SLAP IPs from cells transfected with SLAP Δ C with anti-Lck IPs from cells trans-

fected with an empty vector (Fig. 9). Lack of phosphoproteins in anti-SLAP IPs from cells transfected with vector alone indicates the specificity of the interactions. In addition to the phosphoproteins already identified by GST pulldown experiments (pp24, pp36/38, pp76, and pp95; Fig. 8), we consistently observed three new species in both SLAP and Lck IPs: pp50, pp56, and pp70 (Fig. 9, see arrowheads and bracket). To identify the proteins detected by anti-PTyr blotting, we reprobated the blot with Abs specific for the proteins known to participate in TCR signaling and having similar mobility on SDS-PAGE. pp24 was confirmed to contain CD3 ζ chain, pp50 contained α -tubulin, pp56 contained Lck, pp70 contained ZAP-70, pp76 contained SLP-76, and pp95 contained Vav. pp36/38 is likely to represent LAT, but we could not confirm this by Western blotting with anti-LAT heteroserum, perhaps because of the difficulty in detecting phospho-LAT with this

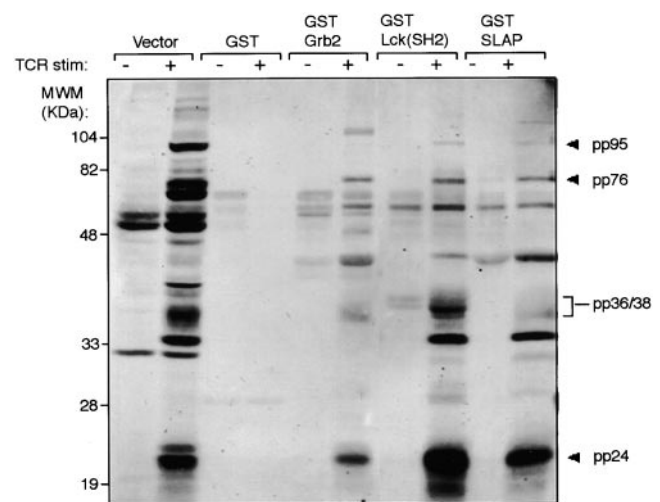


Figure 8. SLAP interacts *in vitro* with a specific subset of phosphoproteins. Lysates from unstimulated (–) or stimulated (+) Jurkat TAG cells were used in the GST pulldown experiment with the indicated fusion proteins (see Materials and Methods). Tyrosine-phosphorylated proteins that interacted with the fusion proteins were resolved by SDS-PAGE and detected with the anti-PTyr mAb (4G10). Soluble WCLs were used as a control for stimulation. Molecular weight markers are indicated on the left (MWM), and the phosphoproteins consistently present in GST-SLAP pulldowns are marked by arrowheads and a bracket on the right.

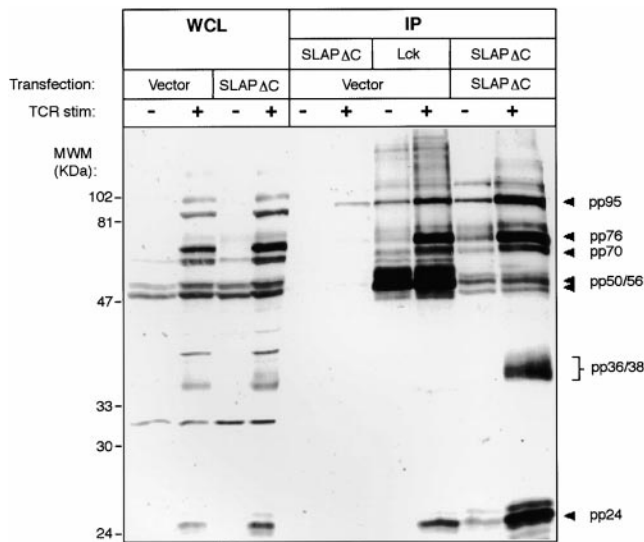


Figure 9. SLAP interacts in vivo with a specific subset of phosphoproteins. Jurkat TAg cells were transiently transfected with either an empty vector or pEF-SLAP Δ C. After 12–16 h, cells were either left unstimulated (–) or stimulated with soluble anti-TCR Ab (C305) for 2 min (+), and lysed in 1% NP-40 lysis buffer. Equivalent amounts of lysates were used in anti-Lck and anti-SLAP IPs (see Materials and Methods). Subsequently, WCLs or washed immunoprecipitates were separated by SDS-PAGE and probed with anti-PTyr mAb (4G10). MWM, molecular weight marker.

reagent. Although pp36/38 and pp50 were predominantly present in SLAP Δ C IPs, we could also detect pp36/38 in Lck IPs on longer exposure of the blot. These results demonstrate that SLAP interacts, both in vitro and in vivo, with many proteins known to participate in TCR signaling.

Discussion

In this study, we found that SLAP can function as a negative regulator of TCR signaling. The T cell is a relevant cell type for studying SLAP function, since SLAP is expressed in T and B lineage cells. Overexpression of SLAP in Jurkat T cells potentially inhibited the TCR-dependent increase in NFAT-driven transcription. Inhibition of AP-1 and IL-2 transcription was also observed. A structure–function analysis revealed that both the SH3 and SH2 domains contribute to this inhibitory function. WT SLAP protein colocalized with the CI-M6PR associated with endosomes, but SLAP mutants with inactive SH2 domain exhibited an altered diffuse, vesicular pattern. SLAP was insoluble in mild detergents, and this property depended on the unique COOH terminus. In stimulated Jurkat cells, SLAP associated with many proteins from the TCR signaling complex. Collectively, these results suggest that SLAP interacts with the stimulated TCR complex to downregulate TCR-mediated signals.

It was originally thought that SLAP RNA is expressed ubiquitously among various tissues (21), and endogenous protein was detected in NIH 3T3 fibroblasts (22). Here, we extended the study of SLAP expression to immune tissues

and several cell lines representing different cell types. We confirm the finding of Pandey et al. (21) that the spleen expresses high levels of SLAP mRNA, but we find that high level of expression is restricted to the immune tissues, with the highest level present in the thymus. In addition, among various cell types tested, only thymocytes, the purified primary T cells and B cells, and a T cell line (EL-4) expressed high levels of SLAP message. Our studies used Northern blots containing 10 μ g of total RNA (Fig. 1) instead of 2 μ g of enriched poly-A messenger RNA (21). Therefore, we could detect only relatively high levels of expression in lymphoid tissues. Unlike the data of Roche et al. (22), who used a heterologous rabbit serum and reported that NIH 3T3 fibroblasts expressed endogenous SLAP protein, we were unable to detect SLAP RNA in NIH 3T3 cells. This difference might be due to clonal variability. However, we would suggest that the high level of SLAP expression is specific to the immune tissues, and that T cells and B cells are predominant cell types expressing it.

Structural resemblance of SLAP to Src family kinases and the recent finding that microinjected SLAP can negatively regulate a Src-dependent receptor signaling pathway of the PDGFR in fibroblasts (22) suggested that SLAP could be a common negative regulator of signaling pathways dependent on activity of Src family members. Since TCR signaling requires Src family (Lck or Fyn) kinase activity (2, 37), we tested the effects of SLAP overexpression in Jurkat cells on TCR signaling. Consistent with the role of SLAP in PDGFR signaling, we find that SLAP overexpression in Jurkat T cells significantly inhibited TCR-induced signal transduction as measured by transcriptional activity of IL-2 promoter and two of its integral elements, NFAT and AP-1. SLAP did not inhibit general transcription in a nonspecific manner, since a constitutively active promoter of Rous sarcoma virus was not affected. These results suggest that the presence of adequate levels of SLAP protein in T cells could render them unresponsive to TCR signaling. Consistent with this hypothesis, we found that Jurkat cells, which respond to TCR stimulation very well, do not express detectable endogenous SLAP protein. However, EL-4 cells, which respond to anti-TCR engagement very poorly, express SLAP at a level sufficient to significantly inhibit NFAT induction when ectopically expressed in Jurkat. Preliminary studies suggest that the level of SLAP protein in T cells may be regulated after transcription. We found that thymocytes, despite expressing high levels of RNA, express very low levels of SLAP protein (data not shown). Moreover, resting T cells, a majority of which can be activated by engagement of their TCRs, express SLAP RNA but no detectable SLAP protein (as determined by IPs followed by Western blotting; data not shown). Stimulation of both populations of cells has little effect on RNA levels, but protein levels are induced. Further studies will be required to understand the exact mechanism responsible for the regulation of SLAP protein levels.

The inhibitory effects of SLAP appear to influence proximal events involved in TCR signaling, since ionomycin- and PMA-mediated stimulation of IL-2-, AP-1-, and

NFAT-directed transcription was insensitive to SLAP inhibition. The ability of SLAP to uncouple TCR signaling from transcriptional activation of NFAT may, in part, be due to reduced levels of TCR-induced calcium fluxes. Though incomplete, an attenuated TCR-induced Ca^{2+} flux was observed in the presence of SLAP. Notably, anti-TCR stimulation in conjunction with PMA, which should bypass the SLAP-imposed blockade of the protein kinase C/Ras pathway, could not restore NFAT-driven transcription (data not shown). This result indicates that TCR signaling did not generate a sufficient calcium signal in the presence of SLAP to synergize with PMA. Interestingly, J14 cells, a Jurkat mutant deficient in SLP-76 expression, displayed a similar partial reduction in TCR stimulation-induced Ca^{2+} flux, and were also unable to upregulate NFAT-driven transcription when stimulated with anti-TCR Ab and PMA (38). Similar epistasis studies using anti-TCR plus ionomycin suggest that SLAP blocks the function of the protein kinase C/Ras pathway (data not shown). However, we have failed to detect any influence of SLAP on the pattern of TCR-induced protein tyrosine phosphorylation (data not shown). This is not a completely surprising finding, since analysis of protein tyrosine phosphorylation in the whole cell lysates (WCLs) is a very crude way to assess the integrity of the TCR signaling pathway. For example, the only difference observed in SLP-76-deficient J14 cells was the lack of tyrosine phosphorylation of the band corresponding to SLP-76 itself, despite the fact that phospholipase C- γ 1 phosphorylation was also impaired (38). Additional studies will be required to determine whether SLAP prevents tyrosine phosphorylation of specific substrates or serves to relocalize them (for example, by targeting them to endosomes).

Our structure-function analysis showed additive contributions of both the SH3 and SH2 domains of SLAP to its inhibitory function, with the SH2 domain having a stronger effect. Even though no functional effect of the COOH terminus of SLAP on TCR signaling was observed, we speculate that this unique 104-amino acid region might play a role in regulating the protein stability since it contains potential proline-, glutamic acid-, serine-, and threonine-rich (PEST) sequences (39), thereby influencing the level of SLAP protein available to regulate the TCR response. Curiously, this unique COOH-terminal part renders SLAP insoluble in mild detergents.

SLAP protein accumulated in perinuclear structures in Jurkat cells. Indirect immunofluorescence analysis of ectopically expressed SLAP in HeLa cells enabled a better resolution of cellular structures and confirmed colocalization of SLAP with an endosomal marker, CI-M6PR. Interestingly, similar localization was reported for an intracellular pool of Lck (35). Because our functional data place SLAP-dependent blockade upstream of effects caused by PMA and ionomycin, we were surprised that, unlike Lck, no plasma membrane staining was observed for SLAP. However, recruitment of SLAP to the plasma membrane might require TCR stimulation, and it would be difficult to detect this in transfected Jurkat cells where only a thin rim of cytoplasm is seen in immunofluorescent microscopic studies. Moreover, the in-

direct immunofluorescence analysis might not be sensitive enough to detect low, yet functionally significant, amounts of SLAP in the plasma membrane. A similar observation was reported for CTL-associated antigen 4 (CTLA-4) protein where only the intracellular, but not the plasma membrane localization was detected by immunofluorescence staining (40). Therefore, like CTL-associated antigen 4, SLAP protein may rapidly shuttle from the plasma membrane to endosomes. Such shuttling to the plasma membrane may depend on TCR signaling. Since the inhibitory effects of SLAP and its localization depended on its SH2 domain, SLAP could play a role in downregulating the stimulated TCR complex.

Having established that SLAP interferes with the proximal events of TCR signaling, and knowing that it structurally resembles Lck, we hypothesized that both proteins could interact with a similar set of targets. Indeed, the pattern of phosphoproteins interacting *in vitro* and *in vivo* with both proteins is very similar. Notably, tyrosine-phosphorylated TCR ζ chain, ZAP-70, SLP-76, and Vav, as well as a 36/38-kD protein which likely represents LAT, were all present in the SLAP-interacting complexes. We were unable to confirm these results with an endogenously expressed SLAP in EL-4 cells, since SLAP protein is insoluble in mild detergents necessary to preserve protein-protein interactions. These data showed that SLAP and Lck can interact with TCR-containing signaling complexes of similar composition.

Since the original submission of this manuscript, another study examining the role of SLAP in T cells was reported by Tang et al. (41). Although both studies used the transient transfection system in Jurkat cells, Tang et al. found that expression of a GST-SLAP fusion protein potentiated TCR-induced NFAT responses, in marked contrast to the inhibitory effects we observed. The apparent difference could relate to the use of a GST fusion of SLAP by Tang et al., rather than epitope-tagged proteins used by us. We confirmed our results by using a different construct encoding a myc-tagged human SLAP (data not shown). Moreover, Tang et al. used only a single dose of GST-SLAP plasmid, whereas we used a broad range of SLAP-encoded plasmid and found a dose-dependent inhibitory effect on IL-2, NFAT, and AP-1 reporters. Nonetheless, despite the differences, our results are in agreement with the findings of Tang et al. that SLAP interacts with a set of proteins relevant to TCR signal transduction.

In conclusion, the data presented here demonstrate that SLAP can function as a negative regulator of TCR function by interfering with the proximal signaling events. Therefore, the differential expression of SLAP protein in T cells could potentially regulate sensitivity to TCR stimulation, thus influencing thymocyte development and mature T cell responses to antigenic stimulation. We are currently testing this hypothesis using SLAP-deficient mice.

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