

Functional Cloning of Src-like Adapter Protein-2 (SLAP-2), a Novel Inhibitor of Antigen Receptor Signaling

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Abstract

In an effort to identify novel therapeutic targets for autoimmunity and transplant rejection, we developed and performed a large-scale retroviral-based functional screen to select for proteins that inhibit antigen receptor-mediated activation of lymphocytes. In addition to known regulators of antigen receptor signaling, we identified a novel adaptor protein, SLAP-2 which shares 36% sequence similarity with the known Src-like adaptor protein, SLAP. Similar to SLAP, SLAP-2 is predominantly expressed in hematopoietic cells. Overexpression of SLAP-2 in B and T cell lines specifically impaired antigen receptor-mediated signaling events, including CD69 surface marker upregulation, nuclear factor of activated T cells (NFAT) promoter activation and calcium influx. Signaling induced by phorbol myristate acetate (PMA) and ionomycin was not significantly reduced, suggesting SLAP-2 functions proximally in the antigen receptor signaling cascade. The SLAP-2 protein contains an NH₂-terminal myristoylation consensus sequence and SH3 and SH2 Src homology domains, but lacks a tyrosine kinase domain. In antigen receptor-stimulated cells, SLAP-2 associated with several tyrosine phosphorylated proteins, including the ubiquitin ligase Cbl. Deletion of the COOH terminus of SLAP-2 blocked function and abrogated its association with Cbl. Mutation of the putative myristoylation site of SLAP-2 compromised its inhibitory activity and impaired its localization to the membrane compartment. Our identification of the negative regulator SLAP-2 demonstrates that a retroviral-based screening strategy may be an efficient way to identify and characterize the function of key components of many signal transduction systems.

Key words: SLAP-2 • SLAP • signal transduction • retrovirus • antigen receptor

Introduction

Antigen receptors on T and B cells recognize pathogens and foreign antigens and initiate a series of intracellular biochemical signaling events that result in complex biological responses (1, 2). These signal transduction events are crucial for T and B cell development as well as T and B cell-mediated immune responses. For example, depending on the developmental stage of the cells, the dosage, type, and duration of the antigen stimulus and the presence of costimulatory signals, engagement of antigen receptors can lead to

either cell proliferation and differentiation or anergy and apoptosis. In the last decade, significant progress has been made toward understanding the mechanism by which antigen receptors initiate such signaling cascades.

In the case of B lymphocytes, the B cell antigen receptor (BCR)* complex consists of ligand-binding Ig heavy and

*Abbreviations used in this paper: a.a., amino acid(s); BCR, B cell antigen receptor; BLNK, B cell linker protein; dox, doxycycline; GFP, green fluorescent protein; IRES, internal ribosome entry site; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; PLC γ , phospholipase C- γ ; SH, Src homology; SLAP, Src-like adapter protein; TRE, tetracycline responsive element; tTA, tetracycline-dependent transactivator.

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light chains, and signal-transducing Ig α and Ig β coreceptors. The engagement of the BCR induces tyrosine phosphorylation of the immunoreceptor tyrosine based activation motifs (ITAMs) present in the cytoplasmic tails of Ig α and Ig β chains and activates multiple cytoplasmic protein tyrosine kinases (PTKs), including Lyn (a Src family kinase), Syk, and Btk (3). Analogous events occur in T cells upon ligand binding to TCR α and β chains. Activation of the Src family kinase Lck leads to tyrosine phosphorylation of the ITAM motifs in the signaling CD3 and TCR ζ chains, resulting in enzymatic activation of ZAP-70. These early biochemical events, in turn, activate a number of downstream effector molecules, including phospholipase C (PLC) γ and guanine nucleotide exchange factors for Ras- and Rho-family GTPases (1, 2, 4). Activation of PLC γ leads to hydrolysis of PIP $_2$ to IP $_3$, which elevates the intracellular Ca $^{2+}$ level, and diacylglycerol, which activates protein kinase C (PKC). Activation of Ras- and Rho-family GTPases regulates cytoskeletal rearrangement, and triggers mitogen-activated protein kinase (MAPK) pathways. The signaling cascades mediated by these secondary effectors ultimately lead to gene transcription in the nucleus and determine the biological outcome of cellular activation.

It has become clear that the integration of distinct signaling cascades required for lymphocyte activation depends on the involvement of specific adaptor proteins (5–7). Adaptors are scaffolding proteins lacking enzymatic domains. However, they contain protein–protein interaction modules such as Src homology (SH) 2 and 3 domains, pleckstrin homology (PH), and WW domains. These modules specifically recognize and bind to discrete protein structures (SH2 domains recognize and bind to phosphotyrosine in the context of a specific flanking sequence; PH domains interact with phospholipids; SH3 domains and WW domains associate with proline-rich regions), thus allowing formation of specific signaling complexes in the cell (8). For example, B cell linker protein (BLNK), a B cell-specific adaptor contains a COOH-terminal SH2 domain and many tyrosine phosphorylation sites (9, 10). After BCR stimulation, the SH2 domain of BLNK interacts with the PTK Syk, which subsequently phosphorylates BLNK. The phosphorylation of BLNK on multiple tyrosine residues leads to recruitment of many critical SH2 domain-containing signaling proteins, including PLC γ , Btk, Vav, and Nck. The importance of BLNK as a key scaffold for connecting different signaling cascades is further underscored by the complete blockage of B cell development and BCR-mediated effector function in both BLNK-deficient mice and cell lines (11–13).

Although significant progress has been made in the past few years in dissecting the early biochemical events initiated by antigen receptors, the exact molecular mechanisms by which these early signaling events ultimately lead to downstream gene activation remain poorly understood. As an improved understanding of such signaling pathways may help to uncover better therapeutic targets for treating transplant rejection and autoimmune diseases, it is of great interest to identify novel key proteins that regulate these complex processes. As an alternative to the traditional

coimmunoprecipitation and protein purification approach, we established a large-scale functional genetic screening method using a retroviral delivery system, which allowed both efficient introduction of cDNAs into B and T cells and regulatable expression of the stably integrated transgenes. After multiple rounds of enrichment of the desired phenotype by fluorescence based sorting, we isolated a cDNA encoding a novel adaptor molecule, named SLAP-2 (for Src-like adapter protein-2). We further showed that overexpression of SLAP-2 markedly impaired antigen-induced surface marker expression and induction of nuclear factor of activated T cells (NFAT) activity. Our data strongly suggest that SLAP-2 may play an important role in negatively regulating T and B cell-mediated effector function. Moreover, our findings suggest that retroviral-based functional screening can be an efficient alternative approach for discovering key regulators in many signal transduction pathways.

Materials and Methods

Cell Culture. Human BJAB B cells, Jurkat T cells (clone N) and Jurkat TAg cells (expressing SV40 large T antigen) were routinely cultured in RPMI 1640 supplemented with 10% fetal calf serum (Hyclone), penicillin, and streptomycin. Phoenix A cells (14) were grown in DMEM supplemented with 10% fetal calf serum, penicillin, and streptomycin. To produce the tetracycline-dependent transactivator (tTA)-BJAB or tTA-Jurkat cell line, BJAB or Jurkat cells were infected with a retroviral construct which constitutively expresses the tetracycline transactivator protein and a reporter construct which expresses LyT2 driven by a tetracycline responsive element (TRE) (15). The tTA-BJAB or tTA-Jurkat cell population was optimized by sorting multiple sounds for high TRE-dependent expression of LyT2 in the absence of doxycycline (dox) and strong repression of LyT2 expression in the presence dox. The cells were also sorted for maximal anti-IgM or anti-TCR-induced expression of CD69. Dox was used at a final concentration of 10 ng/ml for at least 6 d to down-regulate expression of cDNAs from the TRE promoter.

Transfection and Infection. Phoenix A packaging cells were transfected with retroviral vectors using calcium phosphate for 6 h (14). After 24 h, supernatant was replaced with complete RPMI medium and virus was allowed to accumulate for an additional 24 h. Viral supernatant was collected, filtered through a 0.2- μ M filter and mixed with BJAB/Jurkat cells at a density of 2.5×10^5 cells/ml. Cells were spun at room temperature for 3 h at 3,000 rpm, followed by overnight incubation at 37°C. Transfection and infection efficiencies were monitored by green fluorescent protein (GFP) expression and functional analysis was performed 2–4 d after infection. For transient transfection, various amounts of indicated plasmids, plus 10 μ g of NFAT-Luciferase plus 2 μ g of a second control TK Luc luciferase construct (Promega) in the case of the reporter assay, were electroporated into 10^7 BJAB or Jurkat TAg cells, as described previously (16). Cells were cultured for 40 h after transfection and assayed as indicated.

Constructs and Libraries. Dominant negative Δ Syk (amino acid [a.a.] 1–402), SLAP, and SLAP-2 were cloned into the retroviral pTRA-internal ribosome entry site (IRES).GFP vector (17, 18). For generating the myristoylation mutant (SLAP-2-myr), the second a.a. residue, Gly, was substituted with Ala using standard PCR mutagenesis methods. The COOH-terminal trun-

cation mutant (SLAP-2- Δ C) containing a.a. 1–194 was generated by a standard PCR procedure. All three versions of SLAP-2 were COOH-terminal tagged with a FLAG-epitope (DYKDDDDK) and cloned into both the retroviral pTRA-IRES.GFP vector and the mammalian expression vector pEFBOS (16). Sequence similarity was analyzed using MegAlign (DNA*).

RNA extracted from human lymph node, thymus, spleen, and bone marrow was used to produce two cDNA libraries; one random primed and directionally cloned and the second non-directionally cloned and provided with 3 exogenous ATG in 3 frames. cDNAs were cloned into the pTRA-exs vector giving robust dox-regulatable transcription of cDNAs from the TRE promoter. The total combined library complexity was 5×10^7 independent clones.

Stimulation. For CD69 upregulation experiments, tTA-BJAB or tTA-Jurkat cells were split to 2.5×10^5 cells/ml 24 h before stimulation. Cells were spun and resuspended at 5×10^5 cells/ml in fresh complete RPMI medium in the presence of 0.3 μ g/ml anti-IgM F(ab')₂ (Jackson ImmunoResearch Laboratories), 100 ng/ml C305 (anti-Jurkat clonotypic TCR [19]) hybridoma supernatant or 5 ng/ml PMA for 20–26 h at 37°C, and then assayed for surface CD69 expression. For tyrosine phosphorylation and immunoprecipitation, stably infected, GFP-sorted BJAB cells were stimulated with 25 μ g/ml anti-IgM F(ab')₂ at 10^8 cells/ml for 2 min at 37°C in PBS. Cells were then lysed and analyzed.

Cell Surface Marker Analysis. tTA-BJAB or Jurkat-N cells were stained with an allophycocyanin (APC)-conjugated mouse monoclonal anti-human CD69 antibody (Caltag) at 4°C for 20 min and analyzed using a FACSCalibur™ instrument (Becton Dickinson) with CELLQuest™ software. Cell sorts were performed on a MoFlo™ (Cytomation).

cDNA Screen. Phoenix A packaging cells were transfected with a mixture of the two tTA regulated retroviral pTRA-exs cDNA libraries spiked with tTA-regulated pTRA-IRES.GFP- Δ Syk at an estimated 1 in 10^7 copies as an internal positive control. Supernatant containing packaged viral particles was used to infect tTA-BJAB cells with an efficiency of $\sim 80\%$. After 4 d of cDNA expression, library-infected cells were stimulated with 0.3 μ g/ml anti-IgM F(ab')₂ for 20–26 h, stained with APC-conjugated anti-CD69, and lowest CD69-expressing cells were isolated using a fluorescence activated cell sorter. Sorting was repeated over multiple rounds with a 6-d rest period between stimulations until the population was significantly enriched for nonresponders. Single cells were deposited from three separate rounds of sorting. Cell clones were expanded in the presence and absence of dox, stimulated, and analyzed for CD69 upregulation.

Immunoprecipitation and Immunoblots. Stimulated BJAB cells were lysed in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5), protease inhibitors and phosphatase inhibitors, as described previously (16). After 20 min at 4°C, lysates were centrifuged for 20 min at 14,000 rpm and the supernatants were subjected to immunoprecipitation with the indicated antibodies. Resulting immune complexes were washed extensively in lysis buffer, resolved by SDS-PAGE, and transferred to PVDF membranes. Membranes were blocked with 5% albumin in TBST (10 mM Tris, pH 7.8, 150 mM NaCl, with 0.05% Tween detergent). Blots were incubated with the indicated antibodies followed by a secondary antibody conjugated with horseradish peroxidase and then assayed by enhanced chemiluminescence assay (ECL kit; Amersham Pharmacia Biotech). Blots were stripped using 0.1M glycine, pH 2.5, washed extensively in TBST buffer, and then blocked and reprobed as described above. 4G10 monoclonal anti-phosphotyrosine antibodies were from UBI, anti-Cbl

and anti-CD40 were purchased from Santa Cruz Biotechnology, Inc., anti-JNK from Cell Signaling Technology, and anti-FLAG M2 agarose from Sigma-Aldrich.

Luciferase Assays. Transfected BJAB or Jurkat-TAg cells (10^5) were aliquoted into a 96-well plate (Corning) 40 h after transfection and cultured in a final volume of 100 μ l RPMI growth medium. Cells were stimulated at 37°C in the growth medium containing either 1 μ g/ml of C305 hybridoma supernatant or 50 ng/ml PMA and 1 μ M ionomycin. After 12 h stimulation, cells were lysed in 5 \times lysis buffer (Promega) and assayed using the Dual Luciferase reporter kit (Promega). Luciferase activity was read on a Luminometer (Dynex Technology) and determined in triplicates for each experimental condition.

Calcium Mobilization. 10^6 cells were washed in Modified Tyrode's (MT) buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 20 mM Hepes, pH 7.4, 5.6 mM glucose, 0.1% BSA), resuspended in MT at 10^6 cells/ml, and incubated with 4 μ g/ml indo-1 (Molecular Probes) and 4 mM Probenecid (Sigma-Aldrich) at 37°C for 30 min. Cells were centrifuged and resuspended in MT. Analysis was performed on an LSR™ Flow Cytometer (Becton Dickinson) at 37°C. For stimulation, anti-IgM F(ab')₂ was used at 1 μ g/ml, C305 at 300 ng/ml, and ionomycin at 5 μ M.

Cell Fractionation. 10^7 BJAB cells were washed in PBSA followed by hypotonic lysis buffer (HLB: 20 mM Tris/Cl, pH 7.5, 1 mM MgCl₂, 2 mM EGTA, 1 mM DTT, protease and phosphatase inhibitors). Cells were allowed to swell for 20 min on ice followed by lysis by Dounce homogenization. Nuclei were isolated by centrifugation at 1,000 g and discarded. Supernatant was centrifuged at 53,000 g for 40 min. The membrane pellet was washed once with HLB and resuspended in RIPA buffer, followed by dilution 1:1 in HLB. The soluble fraction was diluted 1:1 in RIPA and equal amounts of lysate were assayed.

Results

A Cell-based Assay for Identifying Regulators of Antigen Receptor Signaling. Our goal was to identify cDNA inhibitors of antigen receptor-induced signaling in a B cell line using fluorescence-based sorting of an endogenous cell surface activation marker. After initial evaluation of 10 different cell surface activation markers in 5 human IgM-positive B cell lines, CD69 upregulation in BJAB cells was chosen as the endogenous readout of anti-IgM-induced signal transduction. It has been well established that CD69 upregulation is a prominent early activation event after TCR and BCR stimulation in primary lymphocytes and thus represents a physiological marker for screening (20). To optimize the system for screening, a Tet-off-based tTA gene was stably introduced into the BJAB cells. The tTA system delivers robust transcription of cDNAs from the TRE and enables expression to be switched off using the tetracycline analogue dox (15). The tTA-BJAB cell population was optimized by sorting multiple rounds to achieve maximal anti-IgM-induced CD69 expression and dox-regulatable tTA activity (see Materials and Methods).

The optimized tTA-BJAB cells were further characterized by expressing a dominant negative kinase-deleted version of the B cell signaling protein Syk (Δ Syk). When overexpressed from the TRE enhancer element, Δ Syk significantly reduced anti-IgM-induced CD69 expression

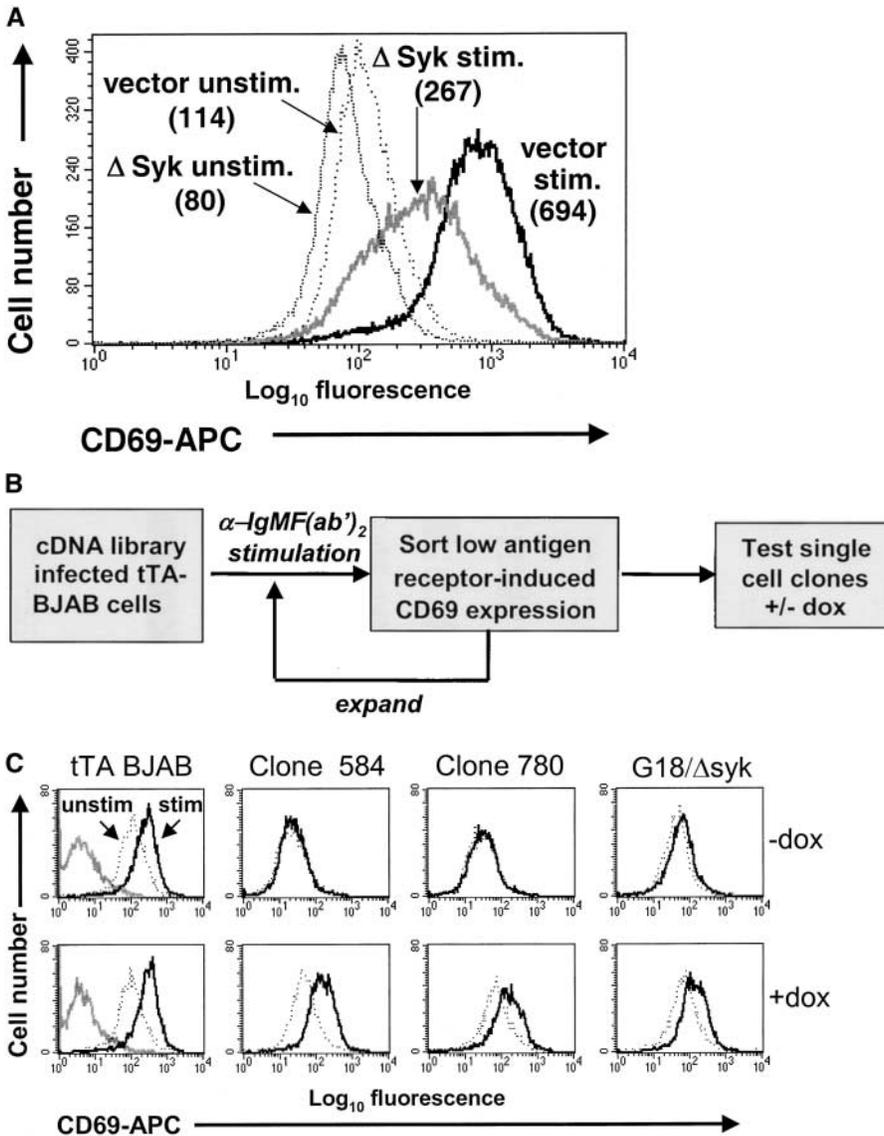


Figure 1. CD69 cell surface marker screen in B-JAB cells. (A) Characterization of the tTA-BJAB cell line using dominant-negative Δ Syk. The tTA-BJAB cell line was infected with pTRA-IRES.GFP vector or a truncated, dominant negative version of Syk (Δ Syk) in the same vector. Cells were stimulated (stim.) with anti-IgM F(ab')₂ and stained for surface CD69 expression. In GFP-gated control cells, fivefold antigen-induced upregulation of CD69 expression over the basal level was achieved. Infection of Δ Syk reduced anti-IgM F(ab')₂-induced CD69 expression to 38% of the control value and also slightly depressed basal CD69 expression. Geometric mean values of CD69 fluorescence are shown in brackets. (B) Screening strategy. tTA BJAB cells stably infected with pTRA cDNA libraries were stimulated with anti-IgM F(ab')₂ and stained for CD69 surface expression. Cells expressing the lowest levels of antigen-induced CD69 were sorted, expanded, and resorted until significant enrichment of nonresponsive cells was observed. Single cell clones were deposited from several sorts and analyzed for anti-IgM F(ab')₂-induced CD69 upregulation in the absence (cDNA on) and presence (cDNA off) of dox. (C) Cell surface CD69 expression in representative positive cell clones. Unstimulated cells (dotted line) and cells stimulated with anti-IgM F(ab')₂ (solid line) were stained with APC-conjugated anti-CD69 antibody. Gray line: stimulated cells stained with isotype control-APC antibody. Inhibition of anti-IgMF(ab')₂-induced CD69 expression (top panel) by cDNAs in clones 584, 780, and G18 (containing spiked control Δ Syk) is reversed when cells are grown in the presence of dox (cDNA off; bottom panel).

(Fig. 1 A). This reduction was sensitive to dox, which suppresses the expression of Δ Syk (data not shown).

Genetic Screen for Regulators of Antigen Receptor Signaling in B Cells. Two TRE-dependent retroviral cDNA expression libraries were constructed using mixed mRNAs extracted from human lymph node, thymus, spleen, and bone marrow tissues. The libraries were pooled, spiked with a tTA-regulated Δ Syk construct as an internal positive control to monitor enrichment of genetic inhibitors, and introduced into tTA-BJAB cells. Cells exhibiting the lowest anti-IgM-induced levels of CD69 expression were enriched by multiple rounds of fluorescence-based sorting and grown out as single cell clones (Fig. 1 B).

A total of 1,394 single cell clones were expanded and plated as duplicates, which were grown in the presence or absence of dox to allow expression of TRE-dependent library cDNAs to be turned off or on. Duplicates were stimulated with anti-IgM F(ab')₂ and assayed for surface CD69 expression. We obtained 128 clones that consistently ex-

hibited a 50% or greater repression of anti-IgM F(ab')₂-induced CD69 expression in the absence (cDNA on) compared with the presence (cDNA off) of dox based on geometric means of the surface CD69 immunoreactivity.

The phenotypes of three representative positive clones (G18, 584, and 780), are illustrated in Fig. 1 C. Sequence analysis demonstrated that clone G18 represents recovery of the dominant negative Δ Syk that was spiked into the library. cDNA sequence analysis of library inserts from additional positive clones identified known negative-regulatory enzymes, including the dual specificity phosphatase 1 which dephosphorylates and inactivates MAPK and diacylglycerol kinase ζ which converts diacylglycerol to phosphatidic acid thus downregulating PKC. In addition, we isolated truncated versions of signaling proteins phospholipase C γ -1, Syk, and a member of the Src family of tyrosine kinases (Hck), which have been shown to be involved in antigen receptor-mediated signaling pathways (data not shown) (1, 2). These cDNAs lack sequences encoding the enzymatic

domains and may act as dominant-negative inhibitors. The isolation of both the spiked Δ Syk and these known antigen receptor regulators confirmed that our screening strategy indeed led to significant enrichment of potent inhibitors of BCR signaling.

Isolation of SLAP and a Novel SLAP Homologue, SLAP-2. Sequencing of retroviral library cDNA inserts recovered from clone 780 identified the Src-like adaptor protein,

SLAP (21). One function previously attributed to SLAP is that of a negative regulator of TCR signaling, although SLAP is also expressed in B cells (22). The isolation of the SLAP cDNA from our screen not only validated the efficacy of the functional selection used, but also established its role in the BCR signaling pathway. Clone 584 contained a cDNA encoding a novel protein of 261 a.a. (Fig. 2 A). Sequence analysis revealed that it shares structural homology

A

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1/1
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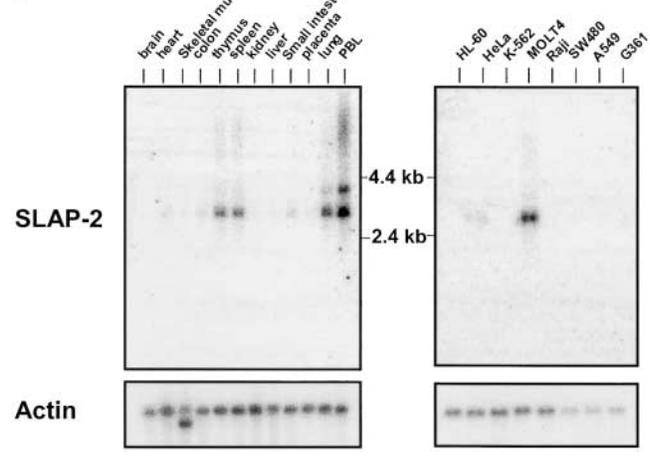
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B

SLAP-2	1	M G S L P S R R K S L P S P S L S S S V Q G G E V T M E A E R S K A T A V A L G S F P A G G P A E L S L R L G E P L T
SLAP	1	M G --- N S M K S T P A P --- A E R E L P N P E G L D S D F L A V I S D Y P S P D I S P P I F R R G E K L R
SLAP-2	61	I V S E D G D W W T V L S E V S G R E Y N I P S V H V A K V S H G W L Y E G L S R E K A E L L L L P G N P G G A F L I
SLAP	51	V I S D E G G N W K A I S L S T G R E S Y I P G I C V A R V Y H G W L F E G L G R D K A E L L L Q L P D T K V G S F M I
SLAP-2	121	R E S Q T R R G S Y S L S V R L S R P A S W D R I R H Y R I H C L D N G W L Y I S P R L T F P S L Q A L V D H Y S E L A
SLAP	111	R E S E T K K C F Y S L S V R R ----- Q V K H Y R I F R L P N N W Y I S P R L T F Q C L E D L V N E Y S E V A
SLAP-2	181	D D I C C L L K E P C V L Q R A G L P L G K D I P L F V T V Q R T P L N W K E L D S S L L F S E A T G --- E S S L
SLAP	165	D G L C C V L T P C L T Q S T A A P A V R A S S S P V I L R Q K T V D W R R V S R L Q E D P E G T E N P L G V D E S L
SLAP-2	237	L S E G L R E S L S F Y I S L N -D E A V S L D D A-----
SLAP	225	F S Y G L R E S T A S Y L S L T S E D N T S P D R K K K S I S L M Y G G S X R K S S F F S P P Y F E D

domains, respectively. The overall amino acid similarity between SLAP-2 and SLAP is 36%. In the SH2 and SH3 domains alone the similarity between SLAP-2 and SLAP is 48% and between SLAP-2 and the Src family kinase Hck is 45%. (C) Northern blot analysis of SLAP-2 expression. Left: human tissues and right: tumor cell lines including promyelocytic leukemia HL-60, HeLa cell S3, chronic myelogenous leukemia K-562, lymphoblastic leukemia MOLT-4, Burkitt's lymphoma Raji, colorectal adenocarcinoma SW480, lung carcinoma A549, and melanoma G361. Blots were stripped and reprobed with an actin probe (bottom panel). (D) RT-PCR of SLAP-2 from purified human primary cells. Specific SLAP-2 primers or control GAPDH primers were used in a standard PCR reaction with cDNA templates obtained from resting (rest.) and activated (act.) CD4⁺ T cells (stimulated with PHA), CD19⁺ B cells (stimulated with pokeweed mitogen), or CD14⁺ monocytes (human blood fractions MTC panel [CLONTECH Laboratories, Inc.]).

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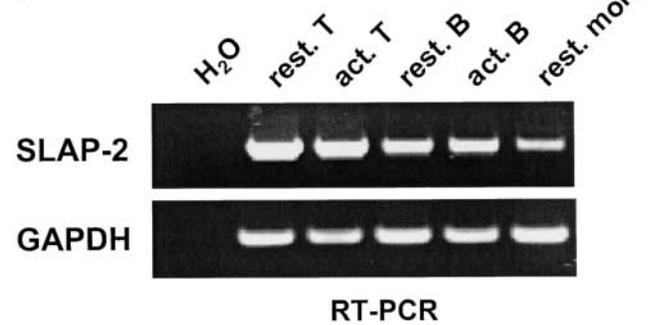


Figure 2. Cloning of a novel inhibitory adaptor, SLAP-2. (A) Human SLAP-2 cDNA sequence. The putative myristoylation site is shown in bold. Open and solid triangles indicate the boundaries of SH3 and SH2 domains, respectively. These sequence data are available from GenBank/EMBL/DDJB under accession no. AF326353. (B) Alignment of human SLAP-2 and SLAP. Identical amino acids are boxed and highlighted. Open and solid triangles indicate the boundaries of SH3 and SH2 domains, respectively. The overall amino acid similarity between SLAP-2 and SLAP is 36%. In the SH2 and SH3 domains alone the similarity between SLAP-2 and SLAP is 48% and between SLAP-2 and the Src family kinase Hck is 45%. (C) Northern blot analysis of SLAP-2 expression. Left: human tissues and right: tumor cell lines including promyelocytic leukemia HL-60, HeLa cell S3, chronic myelogenous leukemia K-562, lymphoblastic leukemia MOLT-4, Burkitt's lymphoma Raji, colorectal adenocarcinoma SW480, lung carcinoma A549, and melanoma G361. Blots were stripped and reprobed with an actin probe (bottom panel). (D) RT-PCR of SLAP-2 from purified human primary cells. Specific SLAP-2 primers or control GAPDH primers were used in a standard PCR reaction with cDNA templates obtained from resting (rest.) and activated (act.) CD4⁺ T cells (stimulated with PHA), CD19⁺ B cells (stimulated with pokeweed mitogen), or CD14⁺ monocytes (human blood fractions MTC panel [CLONTECH Laboratories, Inc.]).

with both SLAP and Src-family kinases, and contains an NH₂-terminal myristoylation consensus sequence (MGX₁₋₄ T/S), one SH2- and one SH3 domain, and a unique COOH terminus, but lacks an enzymatic domain (Fig. 2 B). We therefore named it SLAP-2 for Src-like adapter protein-2. Subsequent analyses of the remaining positive clones identified seven other SLAP-2-containing clones and two more SLAP-containing clones. All of the SLAP-2 and SLAP inserts contained the entire open reading frame and were transcribed in the sense orientation, suggesting that SLAP-2 is a negative regulator.

Northern analysis of multiple tissues indicated that SLAP-2 was predominantly expressed in peripheral blood leukocytes, spleen, thymus, and lung. Abundant SLAP-2 transcript was also detected in the Molt 4 human T cell line, but not other tumor cell lines (Fig. 2 C). Reverse transcription (RT)-PCR analysis revealed that SLAP-2 transcript was present in both BJAB and Jurkat T cell lines, unstimulated and stimulated purified human T cells, B cells, and monocytes, but was absent in fibroblasts and epithelial cells (Fig. 2 D, and data not shown). Thus, SLAP-2 appears to be a hematopoietic-specific inhibitory signaling adaptor.

SLAP-2 Inhibits Antigen Receptor-triggered CD69 Induction and NFAT Promoter Activation in T and B Cells. To confirm that the inhibitory phenotype observed in clones 584 and 780 was indeed dependent on the presence of the cDNAs, full length SLAP-2 and SLAP cDNAs were subcloned into the pTRA-IRES.GFP retroviral vector (17) and reintroduced into parental tTA-BJAB cells (Fig. 3 A). Coexpression of SLAP-2 or SLAP with GFP allowed for parallel analysis of uninfected and infected cells within the same sample, thus providing an internal control. No significant difference in basal CD69 levels was observed between GFP-positive and GFP-negative cells within samples (data not shown). Cross-linking of the BCR with anti-IgM led to a ~3.5-fold upregulation of CD69 expression in both uninfected and infected cells in the vector control population (data not shown). However, in GFP-positive cells expressing either SLAP-2 or SLAP, anti-IgM-induced CD69 expression was significantly reduced compared with vector control or uninfected controls (Fig. 3 A) verifying that the inhibitory phenotype could be transferred to naive cells. In contrast, expression of SLAP-2 did not affect CD69 induction in response to PMA, which bypasses early signaling events leading to activation of PKC and downstream signaling molecules Erk 1/2 MAPKs.

SLAP has previously been shown to inhibit signaling downstream of the TCR (22). As SLAP-2 transcript is observed in T cells, we investigated whether SLAP-2 is also capable of attenuating signals initiated from the TCR. The pTRA-IRES.GFP retroviral vector containing SLAP-2 was reintroduced into tTA-Jurkat cells (Fig. 3 B). No significant differences in CD69 levels were observed between GFP-positive and GFP-negative cells within unstimulated vector control or SLAP-2-infected samples (data not shown and Fig. 3 B). In some experiments, a small decrease in basal CD69 expression was noted in cells expressing SLAP-2

(data not shown). TCR cross-linking using C305 antibody upregulated CD69 expression ~3.5-fold in both GFP-positive and GFP-negative cells in the vector-infected population (data not shown and Fig. 3 B). In contrast, expression of SLAP-2 or SLAP in Jurkat cells strongly repressed C305-stimulated CD69 expression (Fig. 3 B). SLAP-2 expression, however, only marginally affected phorbol ester-induced CD69 levels (Fig. 3 B). Taken together, these data demonstrate that, similar to SLAP, SLAP-2 functions as a negative regulator of antigen receptor-mediated activation events that lead to CD69 upregulation in both T and B cells. Moreover, the observation that SLAP-2 does not significantly block PMA-induced CD69 induction implies that it may function in early signaling events after antigen receptor stimulation.

Antigen receptor activation contributes to the production of many cytokines, including IL-2. Cis-acting elements in the IL-2 promoter bind proteins such as NFAT (23). Reporter constructs containing multimers of the NFAT element are responsive to antigen receptor-mediated signaling in T and B cells and require activation of both the calcium and MAPK pathways (23–27). To determine whether SLAP-2 plays a role in BCR and TCR-triggered NFAT activation, we transiently overexpressed SLAP-2 in BJAB and Jurkat-TAg cells and examined its effect on the NFAT-Luciferase reporter. SLAP-2 transfection into BJAB cells resulted in a decrease in BCR-mediated NFAT-luciferase induction. Similarly, overexpression of SLAP-2 in Jurkat cells led to a reduction of basal NFAT activity as well as an almost complete inhibition of TCR-induced NFAT activation (Fig. 3 C). In contrast, it did not block the NFAT activation in Jurkat cells induced by treatment with phorbol ester and ionomycin, two pharmacological agents that bypass early antigen receptor signaling (Fig. 3 C). To confirm that these inhibitory effects observed were not due solely to pronounced overexpression of the protein, the NFAT assay was repeated, transfecting different quantities of DNA into Jurkat cells to produce a titration of SLAP-2 expression (Fig. 3 D). Both low and high SLAP-2 protein expression repressed anti-TCR-induced NFAT promoter activation, the inhibition being more pronounced at higher SLAP-2 concentrations. A graded inhibitory response was also observed in a similar transient SLAP-2 titration using upregulation of endogenous CD69 as the readout (data not shown). In summary, SLAP-2 appears to play an important receptor-proximal role in downregulating T and B cell-mediated responses.

SLAP-2 Inhibits Antigen Receptor-induced Calcium Mobilization. Cross-linking of the antigen receptor on B or T lymphocytes initiates several well-characterized signaling pathways including the Ras/MAPK pathway, which leads to the phosphorylation of AP-1, and the calcium-dependent pathway that contributes to the activation of NFAT. BJAB and Jurkat cells stably infected with SLAP-2 in pTRA-IRES.GFP or empty vector were stained with the calcium indicator indo-1, stimulated and analyzed on a flow cytometer allowing for simultaneous detection of calcium signals in GFP-negative or -positive cell populations.

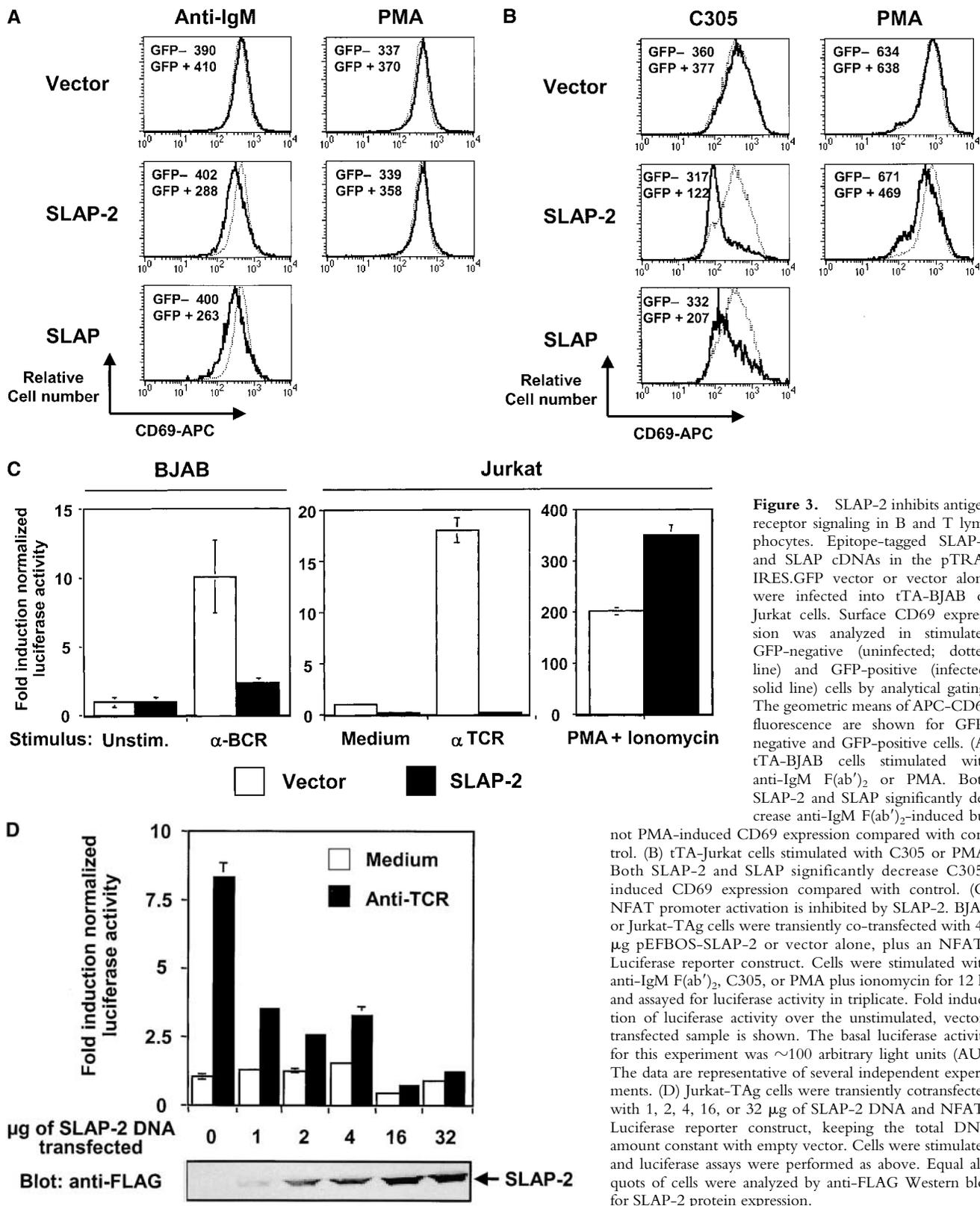


Figure 3. SLAP-2 inhibits antigen receptor signaling in B and T lymphocytes. Epitope-tagged SLAP-2 and SLAP cDNAs in the pTRA-IRES.GFP vector or vector alone were infected into τ TA-BJAB or Jurkat cells. Surface CD69 expression was analyzed in stimulated GFP-negative (uninfected; dotted line) and GFP-positive (infected; solid line) cells by analytical gating. The geometric means of APC-CD69 fluorescence are shown for GFP-negative and GFP-positive cells. (A) τ TA-BJAB cells stimulated with anti-IgM F(ab')₂ or PMA. Both SLAP-2 and SLAP significantly decrease anti-IgM F(ab')₂-induced but

not PMA-induced CD69 expression compared with control. (B) τ TA-Jurkat cells stimulated with C305 or PMA. Both SLAP-2 and SLAP significantly decrease C305-induced CD69 expression compared with control. (C) NFAT promoter activation is inhibited by SLAP-2. BJAB or Jurkat-Tag cells were transiently co-transfected with 40 μ g pEFBOS-SLAP-2 or vector alone, plus an NFAT-Luciferase reporter construct. Cells were stimulated with anti-IgM F(ab')₂, C305, or PMA plus ionomycin for 12 h, and assayed for luciferase activity in triplicate. Fold induction of luciferase activity over the unstimulated, vector-transfected sample is shown. The basal luciferase activity for this experiment was \sim 100 arbitrary light units (AU). The data are representative of several independent experiments. (D) Jurkat-Tag cells were transiently cotransfected with 1, 2, 4, 16, or 32 μ g of SLAP-2 DNA and NFAT-Luciferase reporter construct, keeping the total DNA amount constant with empty vector. Cells were stimulated and luciferase assays were performed as above. Equal aliquots of cells were analyzed by anti-FLAG Western blot for SLAP-2 protein expression.

In comparison to cells infected with vector alone, or GFP-negative cells in the SLAP-2-infected populations, diminished antigen-induced calcium mobilization was observed in GFP-positive BJAB and Jurkat cells expressing SLAP-2

(Fig. 4). Moreover, calcium mobilization induced by the ionophore ionomycin was unaffected. These data suggest that SLAP-2 functions to negatively regulate receptor proximal signaling events which lead to mobilization of

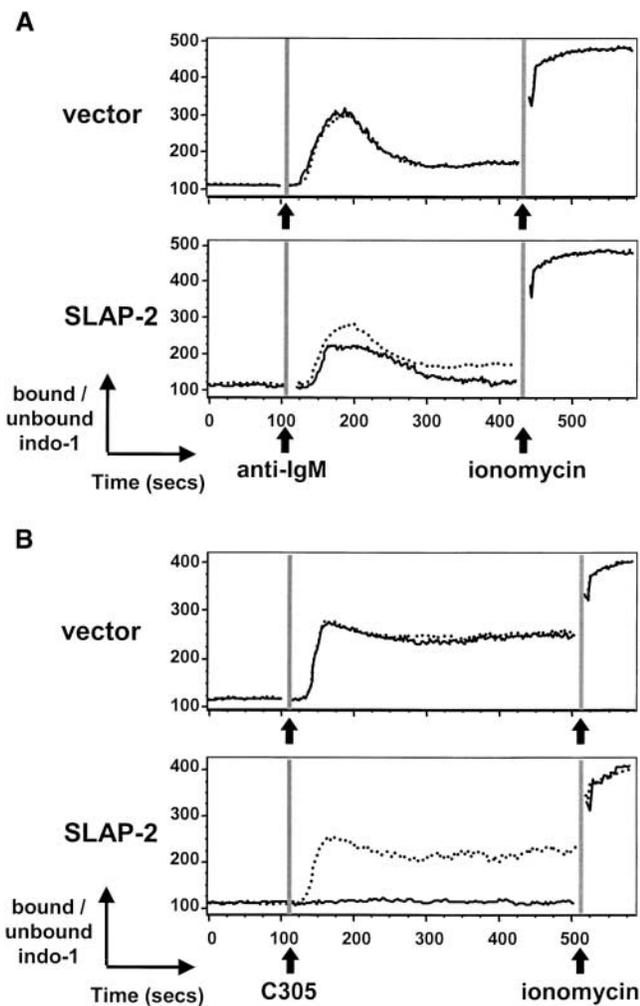


Figure 4. SLAP-2 represses antigen-induced calcium mobilization in BJAB and Jurkat cells. BJAB and Jurkat cells stably expressing SLAP-2 were labeled with the calcium indicator indo-1. Cells were stimulated at 37°C on an LSR flow cytometer with UV laser, and analyzed by analytical gating on GFP. Ratio of bound/unbound indo-1 is shown over a 10-min time course. Broken line: GFP-negative cells; solid line: GFP-positive cells. (A) BJAB cells stimulated with anti-IgM F(ab')₂ followed by ionomycin. (B) Jurkat cells stimulated with C305 followed by ionomycin.

calcium. Although anti-IgM-induced CD69 induction requires predominantly the Ras/MAPK pathway (28), we have been unable to detect modulation of Erk 1/2 MAPK activation by SLAP-2 expression in BJAB cells by Western blotting using phospho-specific anti-activated MAPK antibodies (data not shown). However, this assay may not be sufficiently sensitive to distinguish the small SLAP-2-dependent decreases in MAPK activation that might occur in these cells.

The NH₂-terminal Putative Myristoylation Site and COOH-terminal Region of SLAP-2 Are Important for Inhibition of Antigen Receptor Signaling. Myristoylation has been shown to localize signaling proteins including Src family kinases to the membrane compartment and in many cases to be critical for their function (29). As SLAP-2 contains a putative

NH₂-terminal consensus motif (MGX₁₋₄T/S) for addition of a myristoyl group, we generated an amino acid substitution in the myristoylation motif within a FLAG-tagged full length SLAP-2 (SLAP-2-myr) and examined its effect on antigen receptor-induced signaling. SLAP-2-myr in the pTRA-IRES.GFP vector was introduced into tTA-BJAB cells. The infected GFP-positive cells were sorted and assayed for anti-IgM-induced CD69 expression (Fig. 5 A) and SLAP-2 protein expression (Fig. 5 C). A ~5-fold upregulation of CD69 expression was observed in vector-infected cells after BCR cross-linking (data not shown). Expression of wild-type SLAP-2 suppressed anti-IgM-induced CD69 levels by ~2-fold (Fig. 5 A). In contrast, anti-IgM-induced CD69 expression in cells infected with SLAP-2-myr was comparable to the vector control (Fig. 5 A). Although SLAP-2-myr was expressed at slightly lower levels than wild-type SLAP-2 (Fig. 5 C), the complete loss of activity when the putative myristoylation site was disrupted suggests that this motif may play an important role in SLAP-2 function in BJAB cells. Wild-type SLAP-2 and SLAP-2-myr were also expressed in tTA-Jurkat cells at approximately equal levels (Fig. 5 D) and their effects on TCR signaling were examined (Fig. 5 B). TCR cross-linking stimulated a ~4.5-fold upregulation of CD69 expression in vector-infected cells (data not shown). Expression of wild-type SLAP-2 strongly blocked upregulation of CD69 in response to TCR cross-linking (Fig. 5 B). Once again, mutation of the myristoylation consensus sequence significantly compromised this activity, suggesting that this motif is also important for SLAP-2 function in T lymphocytes.

As myristoylation targets proteins to the membrane, we fractionated the sorted BJAB cells into membrane and cytoplasmic portions and asked where SLAP-2 protein is localized. The purity of membrane and supernatant fractions was confirmed by blotting with antibodies directed against an integral membrane protein (CD40) and a cytoplasmic protein (JNK; Fig. 5 E). A small fraction of epitope-tagged wild-type SLAP-2 was present in the membrane pellet, whereas SLAP-2-myr was almost exclusively cytoplasmic (Fig. 5 E) demonstrating that an intact myristoylation consensus motif is required for association of SLAP-2 with the membrane compartment.

The C terminus of SLAP has been proposed to perform a number of important functions including mediating dimerization, interacting with signaling partners (30), and determining solubility in mild detergents (22). As this region of SLAP-2 exhibits the highest degree of sequence divergence from SLAP, we expressed a truncated form of SLAP-2 (SLAP-2-ΔC) in tTA-BJAB cells and examined its effect on antigen-induced CD69 upregulation. SLAP-2-ΔC-expressing tTA-BJAB cells exhibited anti-IgM-induced CD69 expression comparable to vector control (Fig. 5 A), although some inhibition was observed when the data was gated on the highest GFP-expressing cells (data not shown). SLAP-2-ΔC protein expression was similar to that of wild-type SLAP-2 (Fig. 5 D). This result suggests that although the COOH terminus is important for

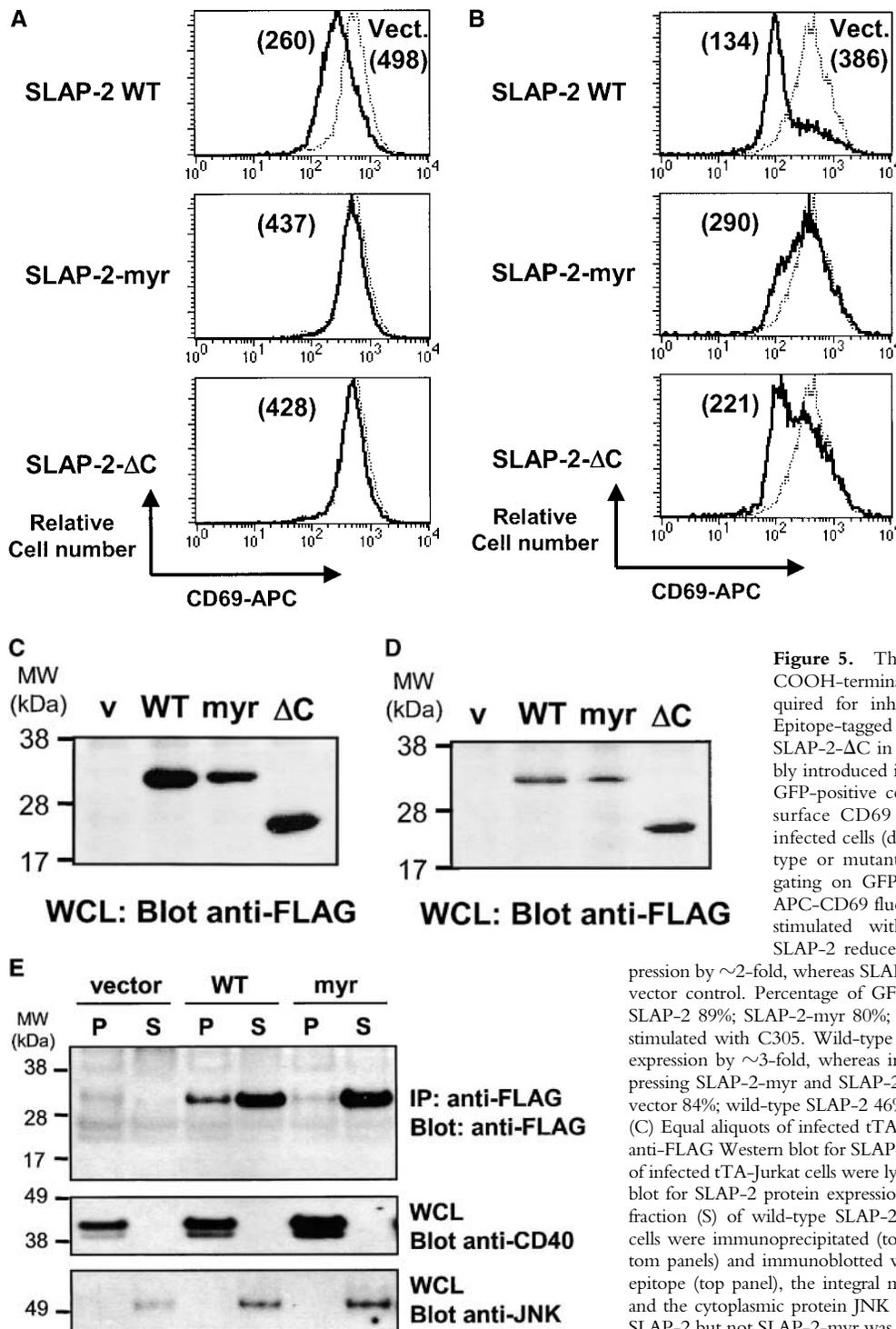


Figure 5. The NH₂-terminal myristoylation site and COOH-terminal unique regions of SLAP-2 are required for inhibition of antigen receptor signaling. Epitope-tagged wild-type SLAP-2, SLAP-2-myr, and SLAP-2- Δ C in the pTRA-IRES.GFP vector were stably introduced into tTA-BJAB or tTA-Jurkat cells, and GFP-positive cells were enriched by sorting. Induced surface CD69 expression was analyzed in vector-infected cells (dotted line) and cells infected with wild-type or mutant SLAP-2 (solid line) with analytical-gating on GFP expression. The geometric means of APC-CD69 fluorescence is shown. (A) tTA-BJAB cells stimulated with anti-IgM F(ab')₂. Wild-type (WT) SLAP-2 reduced anti-IgM F(ab')₂-induced CD69 expression by \sim 2-fold, whereas SLAP-2-myr and SLAP-2- Δ C resembled the vector control. Percentage of GFP positive cells: vector 91%; wild-type SLAP-2 89%; SLAP-2-myr 80%; SLAP-2- Δ C 87%. (B) tTA-Jurkat cells stimulated with C305. Wild-type SLAP-2 reduced C305-induced CD69 expression by \sim 3-fold, whereas inhibition was compromised in cells expressing SLAP-2-myr and SLAP-2- Δ C. Percentage of GFP-positive cells: vector 84%; wild-type SLAP-2 46%; SLAP-2-myr 44%; SLAP-2- Δ C 45%. (C) Equal aliquots of infected tTA-BJAB cells were lysed and analyzed by anti-FLAG Western blot for SLAP-2 protein expression. (D) Equal aliquots of infected tTA-Jurkat cells were lysed and analyzed by anti-FLAG Western blot for SLAP-2 protein expression. (E) Membrane pellet (P) and soluble fraction (S) of wild-type SLAP-2 and SLAP-2-myr-infected tTA-BJAB cells were immunoprecipitated (top panel) or loaded directly (center bottom panels) and immunoblotted with antibodies raised against the FLAG epitope (top panel), the integral membrane protein CD40 (center panel), and the cytoplasmic protein JNK (bottom panel). A fraction of wild-type SLAP-2 but not SLAP-2-myr was localized in the membrane fraction.

SLAP-2 function, its loss can be partially compensated by higher level of protein overexpression.

SLAP-2- Δ C was also less effective than wild-type SLAP-2 at repressing anti-TCR-induced CD69 expression in Jurkat cells (Fig. 5 B). However, a significant level of inhibitory activity was retained. It is possible that the slightly increased protein expression of SLAP-2- Δ C compared with wild-type SLAP-2 may contribute to this effect.

However, cell type-specific variations in SLAP-2-interacting partners or signaling pathways leading to CD69 upregulation may also explain the differences in the efficacy of SLAP-2- Δ C in BJAB and Jurkat cells

SLAP-2 Associates with Tyrosine Phosphorylated Proteins after Antigen Receptor Engagement. Signaling through the TCR or BCR results in a rapid increase in tyrosine phosphorylation of numerous intracellular proteins, initiated

by Src family and Syk/ZAP70 kinase activation (1, 3). These early signaling events ultimately result in transcriptional activation, upregulation of surface antigens, and other lymphocyte effector functions. Adaptor proteins play an important intermediary role in integrating upstream signals to produce biological function (6). Although upon initial evaluation, no changes were discernable in patterns of tyrosine phosphorylation in total cell lysates of BJAB cells overexpressing SLAP-2 (data not shown) we further investigated the nature of SLAP-2 signaling complexes. Epitope-tagged versions of SLAP-2, SLAP-2-myr, or SLAP-2- Δ C were immunoprecipitated from lysates of stably expressing BJAB cells using the anti-FLAG antibody. All three proteins became associated with a number of tyrosine phosphorylated proteins after BCR stimulation (Fig. 6 A), indicating that SLAP-2 indeed participates in BCR signaling pathways. Interestingly, a prominent phosphoprotein of \sim 110 kD was absent in the immunoprecipitates of SLAP-2- Δ C (Fig. 6 A), which we subsequently identified as the RING finger ubiquitin ligase Cbl (Fig. 6 B). Cbl has been previously shown to be a negative regulator of TCR and BCR signaling pathways (31–33). An NH₂-terminal fragment of Cbl constitutively

interacts with the COOH-terminal region of SLAP as demonstrated in both the yeast two hybrid system and Cos-7 cells (30). In contrast, the association between SLAP-2 and full-length Cbl in B cells was inducible after antigen receptor stimulation. Whether SLAP-2 functions as an inhibitory adaptor by recruiting a negative regulator such as Cbl into the signaling complex or by competing with a positive regulator such as a Src-family kinase remains to be determined.

Discussion

In this report, we describe the identification and characterization of a novel adaptor protein, SLAP-2 which contains an NH₂-terminal myristoylation motif and SH2 and SH3 domain protein interaction modules. We demonstrate that SLAP-2 functions as a negative regulator of antigen receptor-mediated signal transduction in both B and T lymphocytes. SLAP-2 shares amino acid and structural homology with the Src-like adaptor protein SLAP, which has been previously reported to downregulate TCR-mediated signal transduction (22). Here, we reveal an additional function for SLAP in repressing signaling through the BCR.

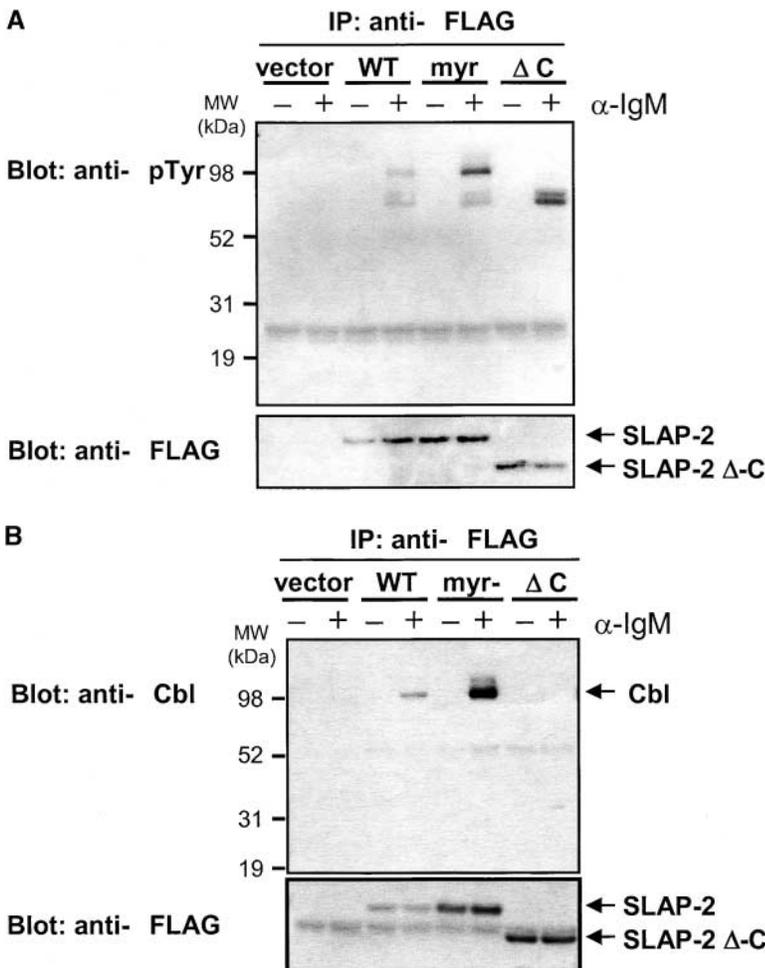


Figure 6. SLAP-2 associates with tyrosine phosphorylated proteins including Cbl after antigen stimulation. (A) SLAP-2 associates with tyrosine phosphorylated proteins in B cells. Sorted τ TA-BJAB cells infected with epitope-tagged wild-type (WT) SLAP-2, SLAP-2-myr, or SLAP-2- Δ C were stimulated with anti-IgM F(ab')₂ for 2 min, lysed, and SLAP-2 was immunoprecipitated using anti-FLAG agarose. Immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies. SLAP-2 associates with tyrosine phosphorylated proteins of \sim 110 and 70 kD after antigen stimulation. The Δ C mutant lacks the 110 kD SLAP-2-associated phosphoprotein. Bottom panel: reprobe with anti-FLAG. (B) SLAP-2 interacts with Cbl in B cells. Wild-type SLAP-2, SLAP-2-myr, or SLAP-2- Δ C were immunoprecipitated as in (A) and immunoblotted with anti-Cbl antibodies. Wild-type SLAP-2 and SLAP-2-myr but not SLAP-2- Δ C associate with Cbl after antigen stimulation.

SLAP-2 and SLAP were isolated in a retroviral-based functional screen for negative regulators of antigen receptor-mediated signal transduction in B cells. This approach was based upon a method previously used to clone the novel anti-apoptosis regulator Toso in T cells (34), and combined an efficient retroviral cDNA delivery system with a FACS[®]-based sorting strategy designed to enrich genetic inhibitors of BCR signaling. The successful isolation of clones containing known modulators of antigen receptor signal transduction pathways validated this approach. Furthermore, the isolation of previously unknown regulators of antigen receptor signaling, including SLAP-2, demonstrates that this strategy can genetically identify novel signaling pathway components based on functional activity in lymphocyte cell lines.

Use of traditional coimmunoprecipitation and biochemical purification methods as well as the yeast two hybrid system have led to the identification of several important kinases and adaptors including ZAP-70, linker for activation of T cells (LAT), BLNK, and SLAP (9, 21, 35, 36). However, physical association in such assays does not provide confirmation of functional significance and cannot distinguish between positive and negative regulators. The generation of somatic mutant cell lines deficient in transduction of antigen receptor-stimulated signals is an alternative functional approach for identifying key components of antigen-receptor signaling pathways. The central role of the T cell signaling components, Lck, ZAP-70, SH2 domain-containing leukocyte protein of 76 kD (SLP-76), and LAT, has been elegantly demonstrated in such mutant lines (37–40). However, random mutagenesis of mammalian cells seldom affects both alleles of a gene, and subsequent cDNA library rescue of such mutant cell lines has been largely unsuccessful. Our functional genetic screening strategy provides a powerful method that complements these conventional target discovery approaches and allows the identification of novel signaling regulators and pathways linked to specific cellular readouts.

Overexpression of either SLAP-2 or SLAP blocks antigen receptor cross-linking-induced upregulation of CD69 in B and T lymphocytes as well as NFAT reporter activation in both cell types (this work and reference 22). In addition, SLAP-2 represses antigen-induced calcium flux in both BJAB and Jurkat cells. It is difficult to conclusively demonstrate that endogenous SLAP-2 functions as an inhibitor of antigen receptor signaling without antisense knockdown or gene targeting experiments. However, we have shown that SLAP-2 acts as a negative regulator of TCR signaling at a wide range of SLAP-2 protein concentrations, suggesting that it may indeed function as an inhibitor at physiological expression levels.

Although the analogous roles of SLAP-2 and SLAP in BJAB and Jurkat cells illustrate the similarities between antigen receptor signaling pathways in B and T lymphocytes, we have observed qualitative differences in the responses in the two different cell types. First, SLAP-2 expression reduces antigen-stimulated CD69 levels more dramatically in Jurkat compared with BJAB cells (28% reduction in BJAB

cells, compared with 61% in Jurkat cells) and also slightly attenuates basal CD69 expression in the T cell line. Correspondingly, expression of SLAP-2 resulted in a more pronounced repression of antigen-induced calcium mobilization in Jurkat than in BJAB cells. Second, experiments using PMA and ionomycin to bypass early antigen receptor signaling events suggest that SLAP-2 acts receptor proximally in both cell types. However a small effect on PMA-induced signaling was observed in Jurkat cells, suggesting that SLAP-2 might also influence more downstream components of the pathway in T cells. Additional mechanistic differences were suggested by the behavior of the SLAP-2- Δ C construct in the two cell types. While the activity of the truncated SLAP-2 was almost completely abrogated in BJAB cells, it retained significant activity in the T cell line suggesting a COOH terminus-independent component to SLAP-2 function in Jurkat cells. Such cell type-specific distinctions in the outcome of SLAP-2 overexpression may reflect differences in SLAP-2 expression levels, the expression level or identity of SLAP-2 effectors, or variation in the potency or composition of signaling pathways employed upstream of CD69 and NFAT upregulation.

The predominantly hematopoietic expression pattern of SLAP-2 resembles that previously reported for SLAP (22). The role of SLAP in antigen receptor signaling is controversial, since it has been described as either a negative (22) or weak positive (30) regulator of TCR signaling using transient reporter assays. Our data using retrovirus-mediated expression and an endogenous cellular marker, strongly suggest a negative regulatory function for SLAP in B cells and SLAP-2 in both B and T lymphocytes. Complementing experiments by Sosinowski et al., we find that overexpression of SLAP in Jurkat cells inhibits anti-TCR-induced CD69 upregulation and calcium flux (Fig. 3 B, and data not shown). Analogously, SLAP-2 effectively inhibits signaling initiated through both the BCR and TCR leading to CD69 upregulation and calcium flux, as well as anti-TCR-induced NFAT activation. Sosinowski et al. report that the SLAP C terminus is dispensable for inhibition of TCR-dependent transcriptional induction from an NFAT reporter construct in a transient overexpression system (22). A similar trend is observed with SLAP-2 when expressed in Jurkat cells where SLAP-2- Δ C inhibitory activity is only modestly compromised. In contrast, truncation of the SLAP-2 C terminus results in an almost complete loss of repression of anti-IgM-induced CD69 upregulation in BJAB cells although this effect may be dependent on SLAP-2- Δ C protein expression level.

Although SLAP-2 and SLAP share structural homologies within their SH2 and SH3 domains, their inhibitory mechanism may in part be different, possibly dictated by unique sequences at the COOH termini of the proteins. In particular, SLAP-2 lacks sequences corresponding to the last 26 a.a. of SLAP, which have been suggested to mediate its dimerization and insolubility in mild detergents, and to negatively regulate interaction of SLAP with the ubiquitin protein ligase, Cbl (22, 30). Indeed, we have experienced no difficulty in solubilizing SLAP-2 in NP-40 lysis buffer, a

condition that reportedly fails to solubilize SLAP (22). Moreover, in contrast to the reported constitutive interaction of SLAP with the N terminus of Cbl (30), we have observed an inducible association between endogenous Cbl and SLAP-2. This disparity may be due to the use of truncated Cbl in these SLAP experiments, or because the *in vivo* coassociation between SLAP and the Cbl N terminus was demonstrated using overexpression in Cos-7 cells (30). The nature of the interaction between SLAP and full length Cbl in lymphocytes remains to be characterized; however, functional differences between SLAP-Cbl and SLAP-2-Cbl interactions are certainly possible.

CD69 induction is predominantly mediated by the Ras/MAPK pathway (28) whereas NFAT activation requires additional input from the calcium-mediated cascade. As SLAP-2 does not significantly inhibit PMA-induced CD69 induction, or NFAT activation in response to PMA plus ionomycin, we propose that the adaptor acts proximal to the receptor complex, upstream of Ras activation and calcium mobilization, as has been suggested for SLAP (22). SLAP-2 effectively inhibits calcium mobilization in Jurkat cells although a more modest effect is observed in BJAB cells. In contrast, initial experiments failed to detect changes in Erk 1/2 MAPK activation in the sorted SLAP-2-expressing BJAB population compared with vector control. However, increasing the sensitivity of the assay by using cells expressing greater amounts of SLAP-2 may allow us to detect inhibition of MAPK activity.

Consistent with the notion that SLAP-2 may act receptor proximally to inhibit antigen receptor signaling, a fraction of wild-type SLAP-2 was associated with the membrane compartment in infected BJAB cells. Moreover, mutation of the NH₂-terminal putative myristoylation sequence abrogated this interaction. Myristoylation is necessary but not always sufficient for stable membrane targeting of signaling proteins (41). Indeed, the localization of SLAP-2 is consistent with the partitioning between cytoplasm and membrane noted upon exogenous expression of the myristoylated SLAP protein (42). We also observed SLAP-2 protein accumulation in the perinuclear region of HeLa cells transiently overexpressing the adaptor, consistent with the localization to endosomes suggested for SLAP (data not shown; 42). Significantly, the integrity of the NH₂-terminal putative myristoylation motif was found to be important for the function of the SLAP-2 in both B and T cell lines. It is possible that myristoylation-mediated localization to the endosomal or plasma membrane may be required to correctly position SLAP-2 close to receptor-proximal signaling proteins.

SLAP is able to associate with phosphorylated tyrosine residues on activated receptor tyrosine kinases (RTKs) (21, 43). Competition with the SH2 domains of Src family kinases for receptor binding sites has been proposed to mediate SLAP inhibition of PDGF-induced mitogenesis in fibroblasts (43). It is possible that SLAP-2 and SLAP may play a similar role in antigen receptor signaling, blocking the interaction of Src family kinases with tyrosine phosphorylated signaling partners. Alternatively, the loss of in-

hibitory function of SLAP-2- Δ C, which correlates with failure to bind to Cbl, suggests that the association of SLAP-2 with the RING finger ubiquitin ligase may contribute to its function. Cbl has been shown to be a negative regulator of Syk/ZAP-70 and Src family kinases (31, 44, 45). One may hypothesize that SLAP-2 and SLAP act like F-box proteins, providing a physical bridge between Cbl and receptor proximal kinases, or may regulate Cbl ubiquitin ligase activity toward specific targets, thereby specifically recruiting them for degradation and resulting in downregulation of antigen receptor-mediated responses. However, the interaction with Cbl is not sufficient for SLAP-2 function, which is independently perturbed by mutation of the myristoylation motif, suggesting that membrane targeting may also be required. Identification of additional SLAP-2-associated proteins will allow a more complete understanding of the mechanism of SLAP-2 function.

Although much recent progress has been made in understanding the positive cellular inputs generated by antigen receptors, increasing evidence indicates that opposing inhibitory mechanisms are also vital to a proper immune response (46). The identification of both SLAP-2 and SLAP from a screen designed to isolate functional inhibitors of BCR signaling strongly suggests that this family of SH2/SH3 domain-containing adaptors plays an important role in immune regulation by attenuating the antigen receptor activation signal in B and T lymphocytes. The dissection of antigen receptor signaling by such strategies may aid in the discovery of improved therapeutic targets for treating transplant rejection and autoimmunity.

We would like to thank our collaborators from Novartis, Jan E. de Vries, and Christoph Heusser for their support during this work. We thank Annette Begrich, Monette Aujay, Mel Fox, and Betty Huang for their excellent technical assistance throughout the screen and Brian Wong for additional experimentation.

Submitted: 16 January 2001

Revised: 20 August 2001

Accepted: 6 September 2001

References

- Weiss, A., and D.R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell*. 76:263–274.
- DeFranco, A.L. 1997. The complexity of signaling pathways activated by the BCR. *Curr. Opin. Immunol.* 9:296–308.
- Bolen, J.B. 1995. Protein tyrosine kinases in the initiation of antigen receptor signaling. *Curr. Opin. Immunol.* 7:306–311.
- Kurosaki, T. 2000. Functional dissection of BCR signaling pathways. *Curr. Opin. Immunol.* 12:276–281.
- Kelly, M.E., and A.C. Chan. 2000. Regulation of B cell function by linker proteins. *Curr. Opin. Immunol.* 12:267–275.
- Tomlinson, M.G., J. Lin, and A. Weiss. 2000. Lymphocytes with a complex: adapter proteins in antigen receptor signaling. *Immunol. Today* 21:584–591.
- Myung, P.S., N.J. Boerthe, and G.A. Koretzky. 2000. Adapter proteins in lymphocyte antigen-receptor signaling.

- Curr. Opin. Immunol.* 12:256–266.
8. Pawson, T., and J.D. Scott. 1997. Signaling through scaffold, anchoring, and adaptor proteins. *Science*. 278:2075–2080.
 9. Fu, C., C.W. Turck, T. Kurosaki, and A.C. Chan. 1998. BLNK: a central linker protein in B cell activation. *Immunity*. 9:93–103.
 10. Wienands, J., J. Schweikert, B. Wollscheid, H. Jumaa, P.J. Nielsen, and M. Reth. 1998. SLP-65: a new signaling component in B lymphocytes which requires expression of the antigen receptor for phosphorylation. *J. Exp. Med.* 188:791–795.
 11. Jumaa, H., B. Wollscheid, M. Mitterer, J. Wienands, M. Reth, and P.J. Nielsen. 1999. Abnormal development and function of B lymphocytes in mice deficient for the signaling adaptor protein SLP-65. *Immunity*. 11:547–554.
 12. Pappu, R., A.M. Cheng, B. Li, Q. Gong, C. Chiu, N. Griffin, M. White, B.P. Sleckman, and A.C. Chan. 1999. Requirement for B cell linker protein (BLNK) in B cell development. *Science*. 286:1949–1954.
 13. Minegishi, Y., J. Rohrer, E. Coustan-Smith, H.M. Lederman, R. Pappu, D. Campana, A.C. Chan, and M.E. Conley. 1999. An essential role for BLNK in human B cell development. *Science*. 286:1954–1957.
 14. Swift, S.E., J.B. Lorens, P. Achacoso, and G.P. Nolan. 1999. Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems. In *Current Protocols in Immunology*, Vol. 10.17C. J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, editors. John Wiley and Sons, Inc., New York. 1–17.
 15. Gossen, M., and H. Bujard. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA*. 89:5547–5551.
 16. Wu, J., S. Katzav, and A. Weiss. 1995. A functional T-cell receptor signaling pathway is required for p95vav activity. *Mol. Cell. Biol.* 15:4337–4346.
 17. Leo, C., Y. Jang, E. Chan, D. Padilla, B.C. Huang, T. Lin, T. Gururaja, Y. Hitoshi, J.B. Lorens, D.C. Anderson, et al. 2001. Dominant effector genetics in mammalian cells. *Nat. Genet.* 27:23–29.
 18. Lorens, J.B., M.K. Bennett, D.M. Pearsall, W.R. Thronset, A.B. Rossi, R.J. Armstrong, B.P. Fox, E.H. Chan, Y. Luo, E. Masuda, et al. 2000. Retroviral delivery of peptide modulators of cellular functions. *Mol. Ther.* 1:438–447.
 19. Weiss, A., and J.D. Stobo. 1984. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J. Exp. Med.* 160:1284–1299.
 20. Ziegler, S.F., F. Ramsdell, and M.R. Alderson. 1994. The activation antigen CD69. *Stem Cells*. 12:456–465.
 21. Pandey, A., H. Duan, and V.M. Dixit. 1995. Characterization of a novel Src-like adapter protein that associates with the Eck receptor tyrosine kinase. *J. Biol. Chem.* 270:19201–19204.
 22. Sosinowski, T., A. Pandey, V.M. Dixit, and A. Weiss. 2000. Src-like adaptor protein (SLAP) is a negative regulator of T cell receptor signaling. *J. Exp. Med.* 191:463–474.
 23. Durand, D.B., J.P. Shaw, M.R. Bush, R.E. Replogle, R. Belagaje, and G.R. Crabtree. 1988. Characterization of antigen receptor response elements within the interleukin-2 enhancer. *Mol. Cell. Biol.* 8:1715–1724.
 24. Emmel, E.A., C.L. Verweij, D.B. Durand, K.M. Higgins, E. Lacy, and G.R. Crabtree. 1989. Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. *Science*. 246:1617–1620.
 25. Woodrow, M., N.A. Clipstone, and D. Cantrell. 1993. p21ras and calcineurin synergize to regulate the nuclear factor of activated T cells. *J. Exp. Med.* 178:1517–1522.
 26. Choi, M.S., R.D. Brines, M.J. Holman, and G.G. Klaus. 1994. Induction of NF-AT in normal B lymphocytes by anti-immunoglobulin or CD40 ligand in conjunction with IL-4. *Immunity*. 1:179–187.
 27. Venkataraman, L., D.A. Francis, Z. Wang, J. Liu, T.L. Rothstein, and R. Sen. 1994. Cyclosporin-A sensitive induction of NF-AT in murine B cells. *Immunity*. 1:189–196.
 28. D'Ambrosio, D., D.A. Cantrell, L. Frati, A. Santoni, and R. Testi. 1994. Involvement of p21ras activation in T cell CD69 expression. *Eur. J. Immunol.* 24:616–620.
 29. Abraham, N., and A. Veillette. 1990. Activation of p56lck through mutation of a regulatory carboxy-terminal tyrosine residue requires intact sites of autophosphorylation and myristylation. *Mol. Cell. Biol.* 10:5197–5206.
 30. Tang, J., S. Sawasdikosol, J.H. Chang, and S.J. Burakoff. 1999. SLAP, a dimeric adapter protein, plays a functional role in T cell receptor signaling. *Proc. Natl. Acad. Sci. USA*. 96:9775–9780.
 31. Rudd, C.E., and H. Schneider. 2000. Lymphocyte signaling: Cbl sets the threshold for autoimmunity. *Curr. Biol.* 10:R344–R347.
 32. Ota, S., K. Hazeki, N. Rao, M.L. Lupher, Jr., C.E. Andoniou, B. Druker, and H. Band. 2000. The RING finger domain of Cbl is essential for negative regulation of the Syk tyrosine kinase. *J. Biol. Chem.* 275:414–422.
 33. Rao, N., M.L. Lupher, Jr., S. Ota, K.A. Reedquist, B.J. Druker, and H. Band. 2000. The linker phosphorylation site Tyr292 mediates the negative regulatory effect of Cbl on ZAP-70 in T cells. *J. Immunol.* 164:4616–4626.
 34. Hitoshi, Y., J. Lorens, S.I. Kitada, J. Fisher, M. LaBarge, H.Z. Ring, U. Francke, J.C. Reed, S. Kinoshita, and G.P. Nolan. 1998. Toso, a cell surface, specific regulator of Fas-induced apoptosis in T cells. *Immunity*. 8:461–471.
 35. Chan, A.C., M. Iwashima, C.W. Turck, and A. Weiss. 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell*. 71:649–662.
 36. Zhang, W., J. Sloan-Lancaster, J. Kitchen, R.P. Tribble, and L.E. Samelson. 1998. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell*. 92:83–92.
 37. Straus, D.B., and A. Weiss. 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell*. 70:585–593.
 38. Williams, B.L., K.L. Schreiber, W. Zhang, R.L. Wange, L.E. Samelson, P.J. Leibson, and R.T. Abraham. 1998. Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. *Mol. Cell. Biol.* 18:1388–1399.
 39. Yablonski, D., M.R. Kuhne, T. Kadlecck, and A. Weiss. 1998. Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T cell. *Science*. 281:413–416.
 40. Finco, T.S., T. Kadlecck, W. Zhang, L.E. Samelson, and A. Weiss. 1998. LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. *Immunity*. 9:617–626.
 41. Resh, M.D. 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim. Biophys. Acta*. 1451:1–16.
 42. Manes, G., P. Bello, and S. Roche. 2000. Slap negatively

- regulates Src mitogenic function but does not revert Src-induced cell morphology changes. *Mol. Cell. Biol.* 20:3396–3406.
43. Roche, S., G. Alonso, A. Kazlauskas, V.M. Dixit, S.A. Courtneidge, and A. Pandey. 1998. Src-like adaptor protein (Slap) is a negative regulator of mitogenesis. *Curr. Biol.* 8: 975–978.
44. Ota, Y., and L.E. Samelson. 1997. The product of the proto-oncogene *c-cbl*: a negative regulator of the Syk tyrosine kinase. *Science.* 276:418–420.
45. Andoniou, C.E., N.L. Lill, C.B. Thien, M.L. Lupper, Jr., S. Ota, D.D. Bowtell, R.M. Scaife, W.Y. Langdon, and H. Band. 2000. The Cbl proto-oncogene product negatively regulates the Src-family tyrosine kinase Fyn by enhancing its degradation. *Mol. Cell. Biol.* 20:851–867.
46. Saito, T. 1998. Negative regulation of T cell activation. *Curr. Opin. Immunol.* 10:313–321.