Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 9775–9780, August 1999
Immunology

SLAP, a dimeric adapter protein, plays a functional role in T cell receptor signaling

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Communicated by Baruj Benacerraf, Dana-Farber Cancer Institute, Boston, MA, June 21, 1999 (received for review March 3, 1999)

ABSTRACT **Engagement of the T cell antigen receptor (TCR) leads to rapid activation of protein tyrosine kinases, which in turn phosphorylate downstream enzymes and adapter proteins. Some adapter proteins, such as SLP-76, Vav, and LAT, positively regulate TCR-mediated signal transduction, whereas others, such as Cbl, play an inhibitory role. SLAP (Src-like adapter protein), an adapter protein containing a Src homology 3 and a Src homology 2 domain, was isolated from a yeast interacting screen by using N-terminal Cbl as bait. N-terminal Cbl interacts with SLAP *in vivo* and *in vitro* in a tyrosine phosphorylation-independent manner. We observed that SLAP is expressed in T cells, and upon TCR activation, SLAP interacts with ZAP-70, Syk, LAT, and TCRζ chain in Jurkat T cells. In transiently transfected COS-7 cells, SLAP forms separate complexes with ZAP-70, Syk, and LAT through its Src homology 2 domain. Overexpression of a C-terminal-truncated SLAP mutant down-regulates nuclear factor of activated T cells-AP1 activity. We have evidence that SLAP forms homodimers through its C-terminal region. Serial truncations and mutations in the C terminus of SLAP demonstrate that there is a correlation between the loss of dimerization and the inhibition of nuclear factor of activated T cells-AP1 activity. The *in vivo* association of SLAP with key signaling molecules and its inhibition of T cell activation suggests that SLAP plays an important role in TCR-mediated signal transduction.**

T cell antigen receptor (TCR)-mediated signal transduction is critical for the development and function of T cells. Engagement of the TCR initiates multiple intracellular events such as hydrolysis of inositol phospholipids, elevation of intracellular calcium, and activation of the Ras/mitogen-activated protein kinase pathway. These signaling events ultimately lead to enhanced lymphokine gene transcription, cellular proliferation, and differentiation (for review, see ref. 1). One of the earliest events in TCR signaling is the activation of Src family kinases. The Src family kinases Lck and Fyn phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) in TCRζ chains. The tyrosine phosphorylation of ITAMs recruits Syk family kinases ZAP-70 and Syk to the TCR, where they are activated through auto/trans-phosphorylation. Syk family kinases in turn phosphorylate several adapter proteins and enzymes (2–5). The adapter protein LAT is one of the most prominent substrates of ZAP-70 and Syk. LAT is a membrane-bound adapter protein that associates directly or indirectly with several crucial signaling molecules, including Grb2, SLP-76, p85 subunit of phosphoinositide 3-kinase (PI3K), and phospholipase Cγ1 (PLCγ1) (6). LAT may recruit PLCγ 1 and PI3K to the membrane, where they function as enzymes and activate calcium and protein kinase C-mediated pathways.

ZAP-70 has been shown to bind Lck (7), Vav (8), SHP-1 (9), and Cbl (10, 11) directly. Both SHP-1 and Cbl are negative regulators of ZAP-70. SHP-1 binds to ZAP-70 through its Src homology 2 (SH2) domain and may reduce ZAP-70 activity by dephosphorylating ZAP-70. The Cbl and ZAP-70 interaction is mediated by the N-terminal phosphotyrosine binding domain of Cbl and phosphorylated tyrosine-292 of ZAP-70 (12).

In a yeast two-hybrid screen used to search for proteins that interact with the N-terminal portion of Cbl (Cbl-N), we isolated a cDNA encoding the C-terminal region of SLAP (Src-like adapter protein). SLAP previously was cloned from another yeast two-hybrid screen by using the receptor tyrosine kinase Eck as bait (13). SLAP is a ubiquitously expressed 34-kDa protein containing a Src homology 3 domain at its N terminus, followed by an SH2 domain and a C-terminal tail without known domain structure. Studies in NIH 3T3 cells indicate that SLAP interacts with the platelet-derived growth factor (PDGF) receptor and negatively regulates mitogenesis (14). In this report, we provide evidence suggesting that SLAP plays an important role in regulating TCR-generated signaling. When Jurkat cells were stimulated with anti-CD3 antibody crosslinking, transiently transfected glutathione *S*-transferase (GST)-SLAP fusion protein associated with ZAP-70, Syk, LAT, and CD3ζ chain through its SH2 domain. A truncation in the C-terminal region of SLAP created a dominant interfering mutation that strongly inhibited TCR-induced nuclear factor of activated T cells (NFAT) activity. Although the C-terminal truncation of SLAP does not compromise its ability to interact with Syk family kinases and LAT, it disrupted the dimerization between SLAP proteins. The data suggest that SLAP is a dimeric adapter protein interacting with several key T-cell signaling molecules and is involved in the TCR-mediated signal transduction pathway.

EXPERIMENTAL PROCEDURES

Plasmids. The cDNA encoding mutant c-src (15) was subcloned into a yeast expression vector p426 (from American Type Culture Collection), which contains the *URA3* selectable marker. The cDNA encoding the N-terminal 25–351 amino acids of Cbl was PCR-amplified and subcloned into the pAS2–1 vector (from CLONTECH) for yeast two-hybrid screen. Wild-type or G306E mutant cDNA encoding the Cbl N-terminal 25–351 region was subcloned into the pGEX 4T-3 (Amersham Pharmacia Biotech) and pEBM vector (provided by B. Mayer, Children's Hospital, Boston) for overexpression in *Escherichia coli* and transient transfection in COS-7 cells, respectively. The full-length SLAP cDNA (from American Type Culture Collection) was subcloned into the pEBG and pEBM vector (provided by B. Mayer), creating GST-SLAP and myc-SLAP, respectively. The full-length SLAP cDNA also was subcloned into pGEX 4T-3 and pET-21a (Novagen) for overexpression of GST-SLAP fusion protein or SLAP protein alone in *E. coli*. SLAP mutants were generated by PCR-directed mutagenesis. In SLAP/RK, arginine-111 was changed to lysine. In SLAP/ΔT and SLAP/RK-ΔT, the C-

Abbreviations: TCR, T cell antigen receptor; SH2, Src homology 2; SLAP, Src-like adapter protein; GST, glutathione *S*-transferase; RAM, rabbit anti-mouse IgG antibody; PDGF, platelet-derived growth factor; Cbl-N, N-terminal Cbl; NFAT, nuclear factor of activated T cells; anti-p-Tyr, antiphosphotyrosine antibody.

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terminal region of SLAP (from amino acids 212 to 276) was deleted from wild-type and RK, respectively. In SLAP/Δ15, SLAP/Δ29, and SLAP/Δ48, 15, 29, and 48 aa were deleted from the C-terminal region of SLAP, respectively. In SLAP/L3S, Leu-218, Leu-224, and Leu-229 were changed to serine. In SLAP/L2Q, Leu-237 and Leu-239 were changed to glutamine. The cDNA encoding LAT (from L. Samelson, National Institutes of Health, Bethesda; ref. 6] was amplified by PCR and subcloned into the pEBB(HA) vector (provided by B. Mayer). The plasmids carrying Lck or hemagglutinin (HA)-tagged Syk cDNA were generously provided by T. Mustelin (La Jolla Institute for Allergy and Immunology, San Diego). The HA-tagged ZAP-70 construct was kindly provided by A. Chan (Washington University School of Medicine, St. Louis). The NFAT-AP1 luciferase construct has been described (16).

Antibodies. The rabbit anti-SLAP polyclonal antibody was raised against full-length GST-SLAP, which was overexpressed in *E. coli* and purified through glutathione-Sepharose. Rabbit antiserum was affinity-purified according to ref. 17. Briefly, SLAP protein overexpressed from *E. coli* was separated by 10% SDS/PAGE and transferred to nylon membrane. The membrane strip carrying the SLAP protein was incubated with rabbit antiserum at 4°C overnight and washed with PBS. The anti-SLAP antibody bound to the membrane was eluted with 0.2 M glycine (pH 2.8) and 1 mM EGTA, and was neutralized with 0.1 vol of 1 M Tris (pH 7.5). The OKT3.14 (anti-human CD3ε), 9E10 (anti-myc) mAb, and the anti-GST rabbit polyclonal antibody were purified in our laboratory. The anti-LAT polyclonal antibody (UpState Biotechnology), anti-Syk mAb (Santa Cruz Biotechnology), anti-ZAP-70 mAb (Transduction Laboratories, Lexington, KY), anti-CD3ζ mAb (Santa Cruz Biotechnology), horseradish peroxidase-linked antiphosphotyrosine mAb (RC20H, Transduction Laboratories), sheep anti-mouse IgG Dynabeads M-280 (Dynal), and polyclonal rabbit anti-mouse IgG antibody (RAM) (Southern Biotechnology Associates) were purchased from commercial sources.

Yeast Two-Hybrid Screen. The host strain PJ69–4A was provided by P. James (University of Wisconsin, Madison) (18). The constructs c-src/p426 and cbl-N/pAS2–1 were transformed into the PJ69–4A reporter strain. The c-src kinase activity and the expression of $GAL4_{BD}$-Cbl-N fusion protein were tested by Western blot analysis of the whole-cell lysates with antiphosphotyrosine antibody (anti-p-Tyr) RC20H and anti-$GAL4_{BD}$ antibody, respectively. A cDNA library from phytohemagglutinin-stimulated human leukocytes (CLONTECH) subsequently was introduced into the reporter strain (PJ69–4A transformed with cbl-N/pAS2–1 and c-src/p426) in a large-scale transformation. About 1,000,000 transformants were screened. The transformants first were selected on His−/Leu−/Trp−/Ura− plates for 15 days, which selected for the activation of *GAL1-HIS3* reporter gene, and then replica-plated to Ade−/Leu−/Trp−/Ura− plates to select for the *GAL2-ADE2* reporter gene. Plasmids carrying the library cDNA were extracted from positive colonies and retransformed into the original strain. Those plasmids that could again activate the reporter genes were sequenced.

Jurkat T Cell Transient Transfections and Activation. Jurkat cells (1×10^7) were suspended in 500 μl RPMI medium 1640, mixed with 20 μg of DNA constructs, and electroporated at 800 μF/250 V by using a BRL electroporator. The transfected cells were further incubated in 25 ml RPMI/10% FCS for 15 hr. Cells were washed and mixed with 1 μg/ml of OKT3.14 antibody and 10 μg/ml of RAM crosslinking antibody, incubated on ice for 20 min, and transferred to 37°C for 2 min. Three transfection samples were pooled for one glutathione-Sepharose precipitation. For the luciferase assays, 1×10^7 Jurkat cells were transfected with 2.5 μg of the NFAT-AP1 firefly luciferase construct, 0.1 μg of thymidine kinase-driven Renilla luciferase construct (Promega), and 20 μg of the experimental constructs. The transfected cells were incubated at 37°C for 15 hr, and 5×10^5 of these cells were transferred to 48-well plates with or without prebound OKT3.14 antibody. The OKT3.14 stimulation was done at 37°C for 6 hr.

COS-7 Cell Transfections. COS-7 cells were transiently transfected as described (19). Briefly, cells were incubated with 2–8 μg of DNA constructs in DEAE-dextran/Chloroquine-containing medium at 37°C for 3.5 hr. After removal of the DNA-containing medium, cells were treated with 10% DMSO/PBS for 2 min at room temperature. Transfected cells were cultured in fresh medium for 36 hr before harvest.

Immunoprecipitations and Immunoblotting. Cells were lysed as described (19). Cell lysates were incubated with glutathione-Sepharose beads or with 1 μg antibody and protein A-Sepharose beads for 4 hr at 4°C. Beads were washed four times, and the bound proteins were subjected to SDS/PAGE. The ECL detection system (Amersham Pharmacia) was used for immunoblotting (20).

Luciferase Assay. The Dual Luciferase Reporter Assay System (Promega) was used, and the assay was performed according to the manufacturer's protocol. Luciferase activities were determined by the Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego). All experiments were done in duplicate.

RESULTS

SLAP Is a Novel Cbl-N Interacting Protein. In an attempt to identify proteins that associate with Cbl-N in a phosphotyrosine-dependent manner, we used a modified yeast two-hybrid system to screen for such interactions. We used a region corresponding to residues 25–351 of Cbl (Cbl-N) as bait and the c-Src kinase as the source of tyrosine kinase. One of the cDNA clones isolated from this screen encoded the C-terminal portion of SLAP, a previously isolated adapter protein. To study the interaction between Cbl-N and SLAP in mammalian cells, we transiently transfected myc-tagged SLAP (myc-SLAP) into Jurkat cells and stimulated the cells with anti-CD3 antibody crosslinking. The nonstimulated and stimulated cell lysates were incubated with GST-Cbl-N protein immobilized on glutathione-Sepharose. GST-Cbl-N coprecipitated SLAP from both nonstimulated and stimulated cell lysates (Fig. 1*A Upper*, lanes 3 and 4), whereas Cbl-N interacted with ZAP-70 only from activated cells (Fig. 1*A Lower*, lanes 3 and 4). Moreover, the Cbl-N G306E mutation did not affect the interaction of Cbl-N with SLAP (Fig. 1*A Upper*, lanes 5 and 6), although this mutation compromised the Cbl-N and ZAP-70 interaction as previously reported (Fig. 1*B Lower*, lanes 5 and 6). Similar results were obtained from cotransfection studies in COS-7 cells. GST-SLAP and myc-Cbl-N (wild type or G306E) were transiently transfected into COS-7 cells. Both wild-type Cbl-N and the G306E mutant coprecipitated GST-SLAP (Fig. 1*B Lower*, lanes 1–3). Cotransfection of Lck dramatically increased GST-SLAP phsophorylation *in vivo* (Fig. 1*B Upper*); however, the increased SLAP phosphorylation did not lead to an increase in the Cbl/SLAP interaction (Fig. 1*B Lower*, lanes 4–6). These data suggest that Cbl-N and SLAP interact in a phosphorylation-independent manner.

SLAP Associates with ZAP-70, Syk, LAT, and CD3ζ upon TCR Activation. SLAP is a recently cloned adapter protein with no known function in TCR signaling. The presence of an SH2 domain in SLAP, along with its endogenous expression in Jurkat T cells (Fig. 2*A*), prompted us to investigate whether SLAP would associate with other signaling molecules in T cells. Affinity-purified anti-SLAP antibody immunoprecipitated several tyrosyl-phosphorylated proteins from H_2O_2-stimulated Jurkat T cell lysates (Fig. 2*B*). Notably, one set of the bands at 36–38 kDa resembled the T cell-specific adapter protein LAT. Upon anti-CD3 antibody crosslinking, anti-SLAP antibody also could immunoprecipitate this 36- to 38-kDa protein (Fig. 2*C*). We further observed that when GST-SLAP was transiently expressed in Jurkat T cells, it interacted with several tyrosine-phosphorylated proteins upon T cell activation (Fig. 3*A*). The glutathione-Sepharose precipitation pattern was similar to immunoprecipi-

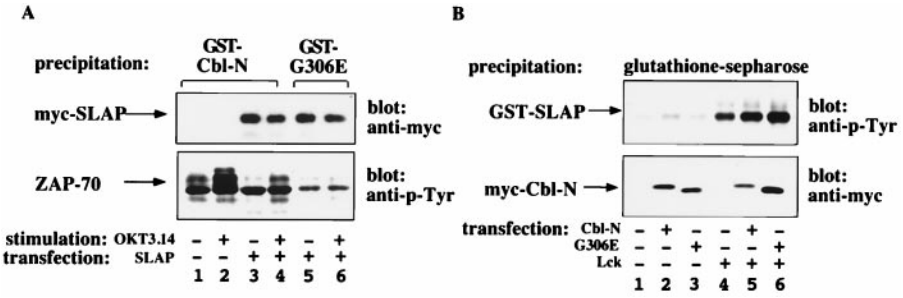

FIG. 1. Cbl-N associated with SLAP *in vivo* and *in vitro* in a tyrosine phosphorylation-independent manner. (*A*) GST-Cbl-N wild-type or G306E mutant proteins were overexpressed in *E. coli* and purified on glutathione-Sepharose. Jurkat cells were transiently transfected with SLAP/pEBM construct and stimulated with OKT3.14 and RAM. Cell lysates were incubated with glutathione-Sepharose-bound GST-Cbl-N and GST-G306E. Protein complexes precipitated by glutatione-Sepharose were separated by 10% SDS/PAGE and blotted with anti-myc and anti-p-Tyr. The positions for transfected myc-SLAP and endogenous ZAP-70 proteins are indicated. (*B*) COS-7 cells were transiently transfected with the constructs indicated. Cell lysates were precipitated with glutathione-Sepharose. Protein complexes were separated by 10% SDS/PAGE and blotted with anti-p-Tyr and anti-myc antibody. The positions for GST-SLAP and myc-Cbl-N are indicated.

tation of endogenous SLAP (Fig. 2*B*). By immunoblotting with specific antibodies, SLAP was found to interact with four major phospho-proteins: Syk, ZAP-70, LAT, and CD3ζ chain (Fig. 3 *B–E*).

SLAP Forms Separate Complexes with ZAP-70, Syk, and LAT Through Its SH2 Domain. To assess whether SLAP forms a multiprotein complex with Syk, ZAP-70, LAT, and CD3ζ chains or interacts with them individually, we studied their interactions in transiently transfected COS-7 cells. Because a Src family kinase is required for ZAP-70 phosphorylation in COS-7 cells (21), we cotransfected Lck, ZAP-70, and GST-SLAP constructs into COS-7 cells (Fig. 4*A*). GST-SLAP coprecipitated ZAP-70 only in the presence of Lck. Because there is not a significant amount of Lck associated with SLAP, Lck may be required only to phosphorylate ZAP-70 rather than to mediate the SLAP/ZAP-70 interaction. A point mutation in the SH2 domain (R111K) of SLAP markedly diminished this interaction, whereas a 64-aa truncation in the SLAP tail region (ΔT) had no effect, indicating that the association between SLAP and ZAP-70 is mediated by the SH2 domain of SLAP. Similar results were obtained with Syk and GST-SLAP cotransfection (Fig. 4*B*). Syk does not require Lck for phosphorylation and activation in COS-7 cells. Phosphorylated Syk interacts with SLAP through the SH2 domain of SLAP. Phosphorylation-dependent interactions between GST-SLAP and LAT also were observed in transiently transfected COS-7 cells (Fig. 4*C*). Again, this interaction required the SH2 domain of SLAP (Fig. 4*D*). Although Syk was used for LAT phosphorylation in this experiment, no significant association between Syk and LAT was observed (data not shown), suggesting that SLAP directly interacts with LAT. The above results indicate that the SH2 domain of SLAP is able to form individual complexes with ZAP-70, Syk, or LAT in a phosphorylation-dependent manner. The SH2 domain-dependent interactions between SLAP and the aforementioned signaling proteins were observed in Jurkat T cells as well. Consistent with our observation in COS-7 cells, the R111K point mutation in the SH2 domain of SLAP abolished its associations with phospho-tyrosyl proteins in activated Jurkat T cells. Meanwhile, the truncation in the SLAP C-terminal region had no effect on SLAP's association with CD3ζ chain, LAT, and the Syk family kinases (Fig. 5).

Dominant Interfering Mutant of SLAP Inhibits TCR-Mediated NFAT-AP1 Activity. One of the downstream effects of TCR activation is the enhancement of cytokine gene transcription. NFAT is one of the major transcription factors that regulates such transcription. To assess the effect of SLAP on NFAT activity, we transiently transfected GST-SLAP into Jurkat T cells along with a luciferase reporter construct under the control of NFAT-AP1 (one of the enhancer elements within the IL-2 promoter). Overexpression of GST-SLAP caused a slight enhancement of TCR-induced NFAT-AP1 activity when compared with the vector control (Fig. 6). Interestingly, the ΔT mutant (a 64-aa truncation in the SLAP C-terminal region) failed to enhance basal NFAT-AP-1 activity and reduced TCR-stimulated NFAT-AP1 activity. Because the ΔT mutant is able to associate with the same phospho-tyrosyl proteins as the wild type, the data suggest that the SLAP/ΔT mutant functions as a dominant interfering mutant that competes with the endogenous SLAP protein for Syk family kinases and/or LAT, leading to the observed NFAT-AP1 inhibition. Indeed, an additional point mutation in ΔT (RK/ΔT) that disrupted its SH2 domain function abolished its inhibition of NFAT-AP1 activity.

SLAP Forms Homodimers Through its C-Terminal Region. In a COS-7 cell transient-tranfection study, we observed that GST-SLAP coprecipitates with myc-tagged SLAP (myc-SLAP) (Fig. 7*A*, lane 1). This interaction does not require Lck or Syk tyrosine kinase activity. We further tested several mutants of SLAP in this dimerization assay. A 15- or 29-aa truncation in the C terminus of SLAP dramatically reduced its dimerization.

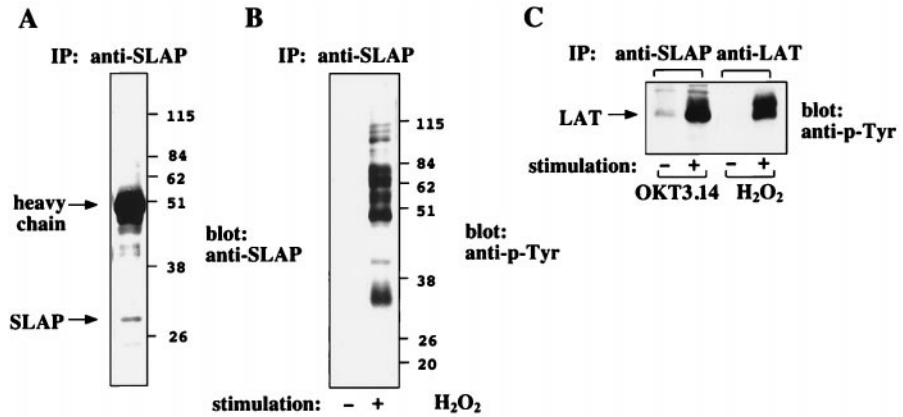

FIG. 2. SLAP is associated with several tyrosyl-phosphorylated proteins in Jurkat T cells. (*A*) Jurkat T cell lysate (from 2×10^7 cells) was incubated with affinity-purified anti-SLAP antibody and protein-A-Sepharose. Immunoprecipitated proteins were separated on 10% SDS/PAGE and blotted with anti-SLAP antibody. The position of SLAP at about 34 kDa is indicated. (*B*) Jurkat T-cells (2×10^7) were stimulated with 5 mM H_2O_2 at 37°C for 3 min. Stimulated and nonstimulated cells lysates were immunoprecipitated with affinity-purified anti-SLAP antibody. Immunoprecipitated proteins were separated on 10% SDS/PAGE and blotted with anti-p-Tyr antibody. (*C*) Jurkat T-cells (5×10^7) were incubated with monoclonal anti-CD3 antibody at 4°C for 10 min. Cells were washed with PBS and incubated with 10-fold sheep anti-mouse antibody conjugated to Dynabeads at 4°C for 10 min. The cell-beads mixture was incubated at 37°C for 3 min and lysed with lysis buffer containing 1% NP-40. Dynabeads were removed from the lysate by centrifugation. Cell lysates were incubated with protein A-Sepharose for 1 hr before the supernatant was immunoprecipitated with affinity-purified anti-SLAP antibody. Protein complexes were separated by 10% SDS/PAGE and blotted with anti-p-Tyr. The anti-LAT immunoprecipitation of H_2O_2-stimulated and nonstimulated lysates were run as a marker for LAT protein.

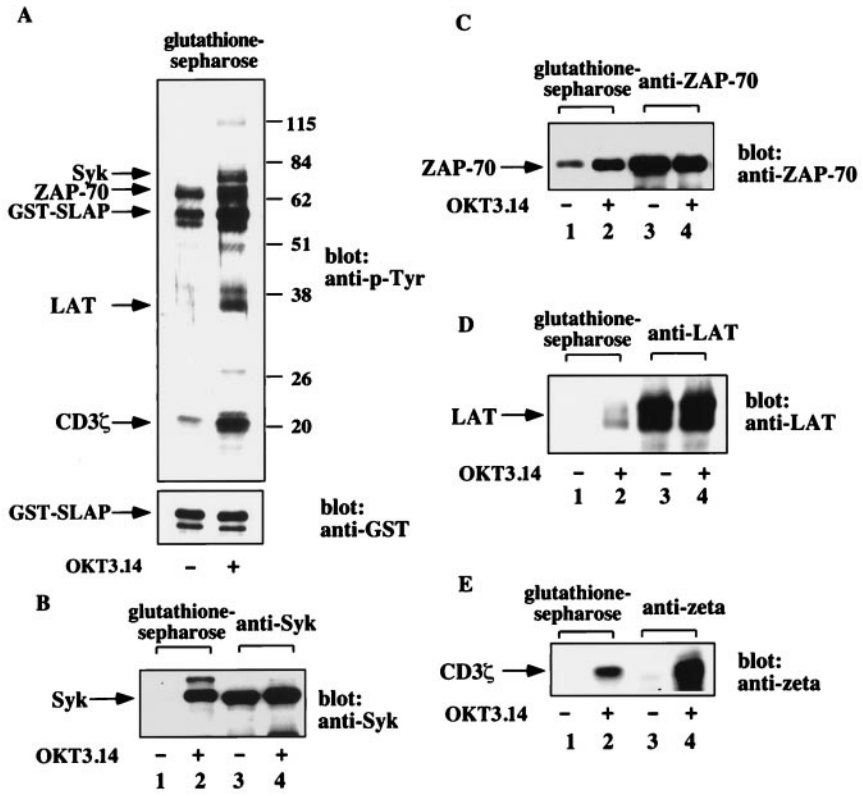

FIG. 3. SLAP associates with ZAP-70, Syk, LAT, and CD3ζ upon TCR activation. (*A*) GST-SLAP was transiently transfected into Jurkat T cells. Fifteen hours after transfection, cells were stimulated by OKT3.14 and RAM antibody at 37°C for 2 min. Cell lysates were incubated with glutathione-Sepharose beads. Bound proteins were separated by 10% SDS/PAGE and blotted with anti-p-Tyr or anti-GST antibody. Potential positions of Syk, ZAP-70, GST-SLAP, LAT, and CD3ζ chain are noted. (*B*) GST-SLAP-transfected Jurkat T cells were activated by OKT3.14 and RAM antibody. Cell lysates were incubated with glutathione-Sepharose beads, and precipitated proteins were blotted with anti-Syk antibody (lanes 1 and 2). Anti-Syk immunoprecipitations of Jurkat T cell lysates were used as a marker for the size of Syk protein (lanes 3 and 4). (*C*) Same as *B*, except anti-ZAP-70 antibody was used for immunoprecipitation and blotting. (*D*) Same as *B*, except anti-LAT antibody was used for immunoprecipitation and blotting. (*E*) Same as *B*, except anti-CD3ζ antibody was used for immunoprecipitation and blotting.

Further truncations of 48 or 64 aa completely eliminated dimerization (Fig. 7*A*, lanes 2–5). These results indicated that SLAP has the potential to form a homodimer through its C-terminal region. There is no known domain structure within the C-terminal region of SLAP. It contains a leucine-rich hydrophobic region (amino acids 210–240) and a highly charged region rich in arginine, lysine, glutamic acid, and aspartic acid (amino acids 240–276) (Fig. 7*B*). Because leucine zippers are well-defined motifs that mediate protein–protein dimerization, we changed several leucine residues in the hydrophobic region. In SLAP/L2Q, Leu-237 and Leu-239 were changed to glutamine. This quite dramatic change of hydrophobic residues to hydrophilic ones only slightly reduced its dimerization with wild-type SLAP (Fig. 7*A*, lane 6). In SLAP/L3S, Leu-218, Leu-224, and Leu-229 were changed to serine. These mutants had no effect on dimerization (Fig. 7*A*, lane 7). Apparently, the highly charged

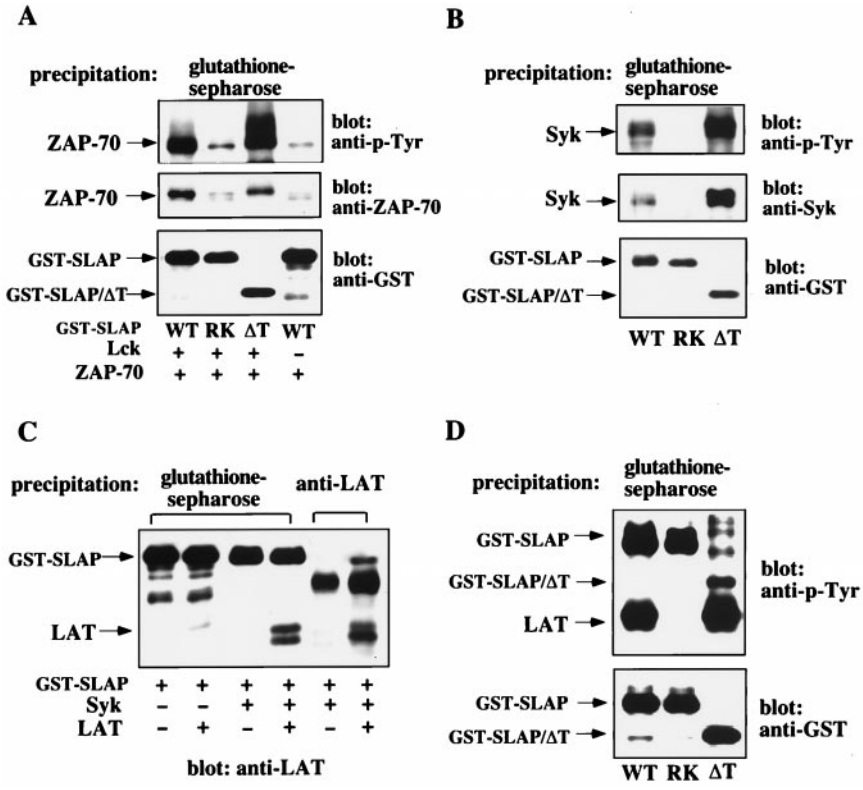

FIG. 4. SLAP forms separate complexes with ZAP-70, Syk, and LAT in COS-7 cells. (*A*) COS-7 cells were transiently transfected with the indicated cDNA constructs. Cells were lysed after 48 hr of transfection. Cell lysates were incubated with glutathione-Sepharose beads. Protein complexes bound to beads were separated by 9% SDS/PAGE and blotted with anti-p-Tyr, anti-ZAP-70, or anti-GST antibodies. (*B*) Same as *A*, except Syk and GST-SLAP constructs were cotransfected. Anti-Syk antibody was used for blotting. (*C*) COS-7 cells were transiently transfected with cDNA constructs as indicated. Cells were lysed after 48 hr of transfection. Cell lysates were incubated with anti-LAT antibody and protein A-Sepharose beads or incubated with glutathione-Sepharose beads. Protein complexes bound to beads were separated by 9% SDS/PAGE and blotted with anti-LAT antibody. Because this blotting antibody was raised against a GST-LAT fusion protein, it detects LAT as well as GST-SLAP. (*D*) COS-7 cells were transiently transfected with Syk, LAT, and wild-type or mutant forms of GST-SLAP. Glutathione bead precipitations were blotted with anti-p-Tyr or anti-GST antibody. WT, wild-type SLAP; RK, SLAP Arg-111 changed to Lys; ΔT, 64-aa deletion at C terminus of SLAP.

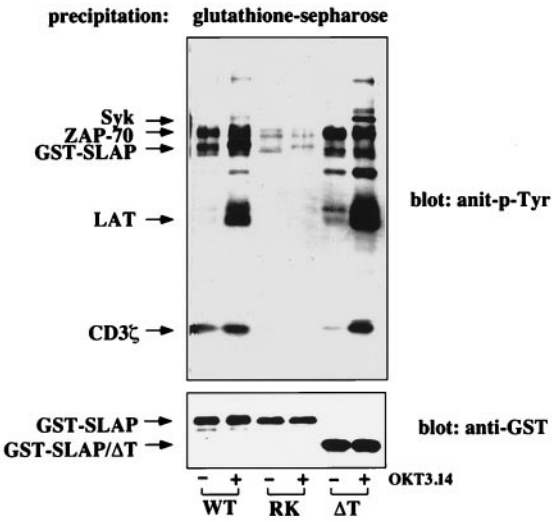

FIG. 5. Activation-dependent interactions between SLAP and phospho-tyrosyl proteins are mediated by the SH2 domain of SLAP. Jurkat T cells were transiently transfected with wild-type or mutant forms of GST-SLAP. Transfected cells were activated with OKT3.14 and RAM antibody at 37°C for 2 min. The activated and nonactivated cells were lysed and incubated with glutathione-Sepharose beads. Precipitated protein complexes were separated by 9% SDS/PAGE and blotted with anti-p-Tyr or anti-GST antibodies. The positions for the known SLAP associating proteins are indicated. WT, wild-type SLAP; RK, SLAP Arg-111 changed to Lys; ΔT, 64-aa deletion at C terminus of SLAP.

region at the C terminus of SLAP protein is responsible for dimerization.

Mutations That Disrupt SLAP Dimerization Also Inhibit TCR-Mediated NFAT-AP1 Activity. To test whether the dominant negative effect of SLAP/ΔT is caused by its lack of dimerization, we overexpressed Δ15, Δ29, Δ48, ΔT, L2Q, and L3S in Jurkat cells and assayed for TCR-mediated NFAT-AP1 activity. The mutants that could not form dimers efficiently (Δ15, Δ29, Δ48, and ΔT) inhibited NFAT-AP1 activity, whereas the wild type and L3S did not (Fig. 8). The L2Q mutant has a modest

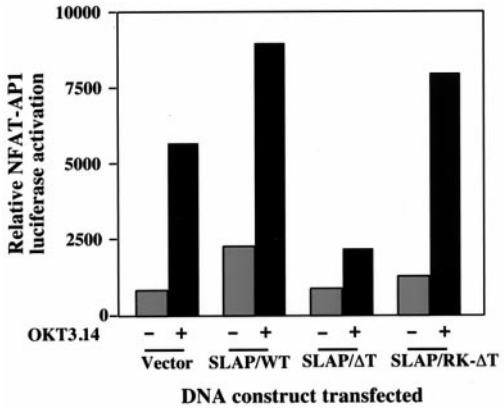

FIG. 6. Dominant interfering mutant of SLAP inhibits TCR-mediated NFAT-AP1 activity. Jurkat T cells were transfected with 2.5 μg of the NFAT-AP1 firefly luciferase construct, 0.1 μg of thymidine kinase-driven Renilla luciferase construct, along with 20 μg of vector control or different GST-SLAP constructs. Transfectants were incubated with or without plate-bound anti-CD3 antibody (OKT3.14) for 6 hr. Cells were lysed, and both firefly luciferase and Renilla luciferase activities were determined according to the manufacturer's instructions. The NFAT-AP1-driven firefly luciferase values were normalized based on the constitutively active Renilla luciferase activity. The data are representative of at least four independent experiments. Vector, pEBG vector; SLAP/WT, wild-type SLAP; SLAP/ΔT, 64-aa deletion at SLAP C terminus; SLAP/RK-ΔT, double mutant of SLAP containing Arg-111 to Lys and 64-aa deletion at C terminus.

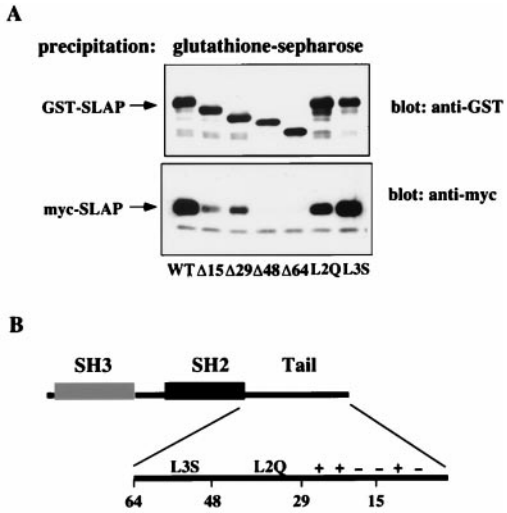

FIG. 7. SLAP forms homodimers in COS-7 cells. (*A*) SLAP/WT, SLAP/Δ15, SLAP/Δ29, SLAP/Δ48, SLAP/ΔT, SLAP/L3S, or SLAP/L2Q in pEBG vector were transiently transfected into COS-7 cells along with myc-SLAP. Cell lysates were incubated with glutathione-Sepharose beads for 4 hr. The protein complexes precipitated on the beads were separated by 9% SDS/PAGE and blotted with anti-GST and anti-myc (9E10) antibody. WT, wild-type SLAP; Δ15, 15-aa deletion at SLAP C terminus; Δ29, 29-aa deletion at SLAP C terminus; Δ48, 48-aa deletion at SLAP C terminus; ΔT, 64-aa deletion at SLAP C terminus; L3S, Leu-218, Leu-224, and Leu-229 were changed to serine; L2Q, Leu-237 and Leu-239 were changed to glutamine. (*B*) A schematic of the SLAP primary structure with the C-terminal tail region shown in more detail. The relative position of truncations and mutations are indicated. + represents positively charged residues such as arginine and lysine. − represents negatively charged residues such as glutamic acid and aspartic acid.

effect on NFAT-AP1 activity, corresponding to its modest effect on dimerization. These data suggest that dimerization is important for SLAP's function in TCR-mediated signaling.

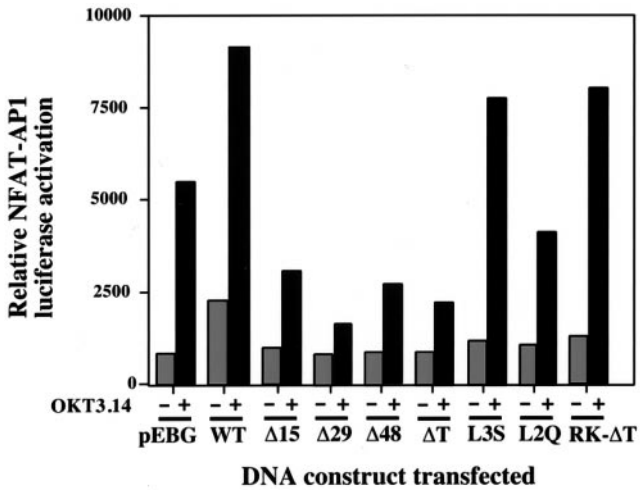

FIG. 8. Mutations that disrupt SLAP dimerization also inhibit TCR-mediated NFAT-AP1 activity. Jurkat T cells were transfected with 2.5 μg of the NFAT-AP1 firefly luciferase construct, 0.1 μg of thymidine kinase-driven Renilla luciferase construct, along with 20 μg of vector control or different GST-SLAP constructs. Transfectants were incubated with or without plate-bound anti-CD3 antibody (OKT3.14) for 6 hr. Cells were lysed, and both firefly luciferase and Renilla luciferase activities were determined according to the manufacturer's instructions. The NFAT-AP1-driven firefly luciferase values were normalized based on the constitutively active Renilla luciferase activity. The data are representative of at least three independent experiments. Refer to Fig. 7*A* for abbreviations of SLAP mutants.

FIG. 9. Cbl-N binding site is mapped to a hydrophobic region in SLAP C terminus. COS-7 cells were transiently transfected with Cbl-N/pEBM and various SLAP wild-type or mutant constructs in the pEBG vector. Cell lysates were precipitated with glutathione-Sepharose. Protein complexes were separated by 10% SDS/PAGE and blotted with anti-GST and anti-myc antibody. The positions for GST-SLAP and myc-Cbl-N are indicated. Refer to Fig. 7*A* for abbreviations of SLAP mutants.

Cbl-N Binding Site on SLAP Is Mapped to the C-Terminal Hydrophobic Region. From the yeast two-hybrid data, we knew that Cbl-N interacts with the C-terminal portion of SLAP. To further define the binding site of Cbl on SLAP, we transfected the SLAP C-terminal truncation and mutation constructs into COS-7 cells along with myc-Cbl-N. Truncation of 15 or 29 aa at the C-terminal region of SLAP increased the SLAP-Cbl-N interaction (Fig. 9). Further truncations into the hydrophobic region abolished the interaction. Two mutations in the hydrophobic region, L3S and L2Q, also abolished the SLAP/Cbl-N interaction. These data suggest that Cbl-N binds to SLAP C-terminal hydrophobic region, instead of the highly charged region.

DISCUSSION

In this report, we demonstrate that the adapter protein SLAP functions in the TCR-mediated signal transduction pathway. The SH2 domain of SLAP is responsible for its association with Syk, ZAP-70, LAT, and CD3ζ chains upon TCR stimulation. The previously uncharacterized tail region mediates homodimerization of SLAP and is important for SLAP's functions in TCR-mediated NFAT-AP1 activation.

A cDNA encoding the C-terminal portion of SLAP was isolated from a yeast two-hybrid screen by using Cbl-N as bait. Because tyrosine kinase c-Src was present in the yeast host strain, the interaction between Cbl-N and SLAP in yeast originally was thought to be mediated by phosphotyrosine binding. Subsequent studies *in vivo* and *in vitro* indicated that the interaction between Cbl-N and SLAP does not depend on tyrosine phosphorylation of SLAP proteins. More interestingly, the well-defined point mutation in Cbl, G306E, disrupted the binding of Cbl-N to ZAP-70 but did not diminish the Cbl-N and SLAP interaction. The three-dimensional structure of Cbl-N has been solved recently (22). It contains a four-helix bundle, a calcium binding EF hand, and a divergent SH2 domain. The SH2 domain, in which the G306E mutant presides, is responsible for most of the interactions with ZAP-70-specific phosphopeptide, whereas the A-B loop in the four-helix bundle helps to form the binding pocket. Because the Cbl-N and SLAP interaction does not depend on G306E or tyrosine phosphorylation, it may represent a new form of interaction mediated by the four-helix bundle or EF hand in Cbl-N.

The binding site of Cbl-N involves the hydrophobic region in the C terminus of SLAP. Note the 15- and 29-aa truncations in the SLAP C terminus enhanced Cbl-N binding dramatically (Fig. 9), whereas these truncations disrupted SLAP homodimerization (Fig. 7). This observation suggests that Cbl-N binds more efficiently to SLAP when SLAP is a monomer. The competition between Cbl-N binding and SLAP homodimerization suggests that Cbl-N binding may represent a mechanism to regulate SLAP dimerization.

SLAP previously has been shown to interact with the tyrosine kinase receptors Eck and PDGF (13, 14). We demonstrate that SLAP functions downstream of the TCR signaling. It is not surprising to find a ubiquitously expressed adapter protein involved in more than one receptor signaling pathway. Cbl, for instance, negatively regulates both PDGF receptor and TCR pathways.

We failed to detect any significant association between SLAP and CD3ζ chain in transfected COS-7 cells (data not shown), nor did we observe much CD3ζ chain coimmunoprecipitated with endogenous SLAP (Fig. 2*B*). TCR, unlike the Eck and PDGF receptors, does not possess a tyrosine kinase domain and relies on the recruited cytoplasmic tyrosine kinases such as ZAP-70 to mediate its function. If SLAP functions similarly in both PDGF receptor and TCR signaling, it is not surprising that it associates with TCR by interacting with ZAP-70. The activation-dependent association between GST-SLAP and CD3ζ chain observed in Jurkat T cells could be mediated by an indirect interaction through ZAP-70.

The effects of wild-type and dominant interfering mutants of SLAP on TCR-mediated NFAT-AP1 promoter activation suggest that SLAP plays a functional role in T cell signal transduction pathways. Moreover, these data suggest that the C-terminal region is critical for SLAP's function. Our observation that the C-terminal region of SLAP facilitated homodimer formation supports our assertion that SLAP-mediated NFAT-AP1 regulation requires the C-terminal region. Without dimerization, SLAP/ΔT serves as a dominant interfering mutant that binds to its target but fails to exert its function. Dimerization events frequently are used in signal transduction pathways. For example, dimerization of CD3ζ may facilitate intermolecular interactions between associated ZAP-70 kinases, and the phosphorylation-dependent dimerization of STAT family proteins may regulate their function as transcription factors (for review see ref. 23). In the case of SLAP, the dimerized adapter protein will possess two SH2 domains and two Src homology 3 domains. The dimer could serve as a bridge between two different molecules or between two of the same molecules. SLAP dimers thus may play a role in the regulation of the activity of Syk family kinases or link proximal signaling molecules to downstream molecules.

We thank Dr. Joanne C. Pratt for critical review of the manuscript. This work is supported by a Postdoctoral Training Fellowship in Cancer Immunology, Dana-Farber Cancer Institute/National Institutes of Health to J.T. and by Grant CA70758 from the National Institutes of Health to S.J.B.

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