A Novel Src Homology 2 Domain-containing Molecule, Src-like Adapter Protein-2 (SLAP-2), Which Negatively Regulates T Cell Receptor Signaling

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We have cloned a novel adapter protein containing Src homology 2 and Src homology 3 domains similar to the Src family of tyrosine kinases. This molecule lacks a catalytic tyrosine kinase domain and is related to a previously identified protein, Src-like adapter protein (SLAP), and is therefore designated SLAP-2. Northern blot analysis indicates that SLAP-2 is predominantly expressed in the immune system. Jurkat T cells express SLAP-2 protein and overexpression of SLAP-2 in these cells negatively regulates T cell receptor signaling as assessed by interleukin-2 promoter or NF-AT promoter reporter constructs. Mutational analysis revealed that an intact SH2 domain of SLAP-2 is essential for this inhibitory effect, whereas mutation of the SH3 domain alone has no effect. This inhibitory effect is upstream of the activation of Ras and increase in intracellular calcium levels, as no inhibition was observed when the cells were activated by phorbol ester plus ionomycin. SLAP-2 interacts with Cbl in vivo in a phosphorylation independent manner and with Zap-70 and T cell receptor ζ chain upon T cell receptor activation. Finally, we show that the mutation of a predicted myristylation site within the NHL-terminal of SLAP-2 is essential for its inhibitory effect. This report therefore implicates SLAP and SLAP-2 as a family of adapter proteins that negatively regulate T cell receptor signaling.

One of the main mechanisms of signaling from the plasma membrane to the nucleus is through the alteration of phosphorylation states of target proteins. Binding of ligands to cellular receptors leads to the activation of the intrinsic or associated kinase activity of receptors in most cases. Phosphorylation of the receptor itself or of its substrates creates docking sites for other proteins within the cells. Such proteins may possess an enzymatic activity such as protein kinases and phosphatases or function as adapter or docking proteins that recruit signaling molecules either by forming signaling complexes or by changing their subcellular localization (1). Adapter proteins contain a variety of modular domains that mediate protein-protein interactions. Some of the best characterized domains involved in protein-protein interactions are Src homology domain 2 (SH2) and Src homology 3 (SH3) domains (2–5). The SH2 domain interacts with phosphotyrosine residues within the context of three to five additional COOH-terminal residues (6). SH3 domains bind to peptides with a left-handed polyproline type II helix containing a minimal consensus sequence Pro-X-Pro (for review, see Ref. 7). Several adapter proteins contain both SH2 and SH3 domains that permit association with multiple binding partners.

T cell activation is a critical event for maturation in the thymus and initiating mature responses in immune cells (8). The T cell receptor (TCR) is a multimeric protein complex formed by the assembly of α and β (or γ and δ) and ε, and ζ CD3 subunits that signals through associated cytoplasmic protein-tyrosine kinases (9–11). Four classes of non-receptor protein-tyrosine kinases, Src, Zap-70/Syk, Tec, and Csk, have been shown to be activated upon immunoreceptor stimulation (12, 13). Activation of these kinases leads to a dramatic increase in phosphorytrosine content of activated cells. Another consequence of T cell activation is the production of IL-2 and other cytokines. The activity of IL-2 promoter is increased by association to specific sites of nuclear factors of activated T cells (NF-AT), AP-1, and members of the NF-κB family by transcriptional up-regulation and stabilization of the mRNA (for review, see Ref. 14).

A number of adapter proteins have been isolated that are involved in TCR signaling; some of them have positive regulatory functions such as LAT, SLP-76, SLAP65, and Gads, while others negatively regulate these signals e.g. SLAP, Dok, and

References

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The abbreviations used are: SH2, Src homology 2; EST, expressed sequence tag; IL, interleukin; NF-AT, nuclear factors of activated T cells; PMA, phorbol 12-myristate 13-acetate; SLAP, Src-like adapter protein; SH3, Src homology 3; TCR, T cell receptor; RT, reverse transcriptase; GST, glutathione S-transferase.
SLAP-2 as a Negative Regulator of T Cell Receptor Signaling

Cbl (15). We have cloned a novel adapter molecule containing SH2 and SH3 domains designated Src-like adapter protein 2 (SLAP-2), whose SH2 and SH3 domains are homologous to the Src family of kinases. SLAP-2 transcript is expressed predominantly in the immune system. In this report, we have explored the function of SLAP-2 and show that it negatively regulates TCR signaling in Jurkat T cells. The SH2 domain of SLAP-2 was critical for this inhibition as evidenced by mutagenesis experiments. Our studies implicate SLAP and SLAP-2 as a family of adapter proteins that negatively regulate signal transduction pathways.

EXPERIMENTAL PROCEDURES

cDNAs and Constructs and Data Base Searches—A mouse EST clone (IMAGE accession number 478854) was obtained from Incyte Genomics (Palo Alto, CA) and sequenced on both strands using ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, CA). A human clone (GenBank™ accession number AK025645) was obtained from Dr. Sumio Sugano (NEDO human cDNA sequencing project). To generate a wild-type SLAP-2 expression vector with a carboxyl-terminal V5 epitope tag, the open reading frame of mouse SLAP-2 was first subcloned into NcoI and XhoI sites of pENTR4 (Invitrogen, Carlsbad, CA) by standard PCR procedures using the primers: aacacctgagaggtctagcaggg (5’ primer), gagagatcagcagggagctgcag (3’ primer) and subsequently, it was transferred into the expression vector pEF/V5-His (Invitrogen) which was modified to be compatible to the Gateway™ system (Invitrogen, Gaithersburg, MD) according to the manufacturer’s instructions.

We used SLAP-2 in pENTR4 as template to obtain point mutations in the SH3 or SH2 domains of SLAP-2 and to mutate the glycine in the position 2 to alanine, using a single primer method (16). The primers containing the mutations were confirmed by sequencing and were subsequently transferred into Gateway™ plasmid pHF5/His to create V5-tagged mutant expression vectors. IL-2 luciferase and NF-AT luciferase reporter constructs were a kind gift from Tomasz Sosinoski and Arthur Weiss. JGEX 4T3 Cbl-N vector has been previously described (17).

The human and mouse SLAP-2 cDNAs were used to search the publicly available human genomic database and Celera’s proprietary mouse genomic database (www.celera.com), respectively. The genomic regions were then aligned to the cDNA sequences to determine intron-exon boundaries.

Northern Blot Analysis—Human multiple tissue and immune system Northern blots containing immobilized poly(A)+ RNA were obtained from CLONTECH (Palo Alto, CA). We used a 740-bp SLAP-2 fragment obtained by AvrII/HindIII digestion of the cDNA as a probe. The probe was labeled with [32P]-labeled dCTP and hybridized according to the manufacturer’s instructions. After autoradiography, the nitrocellulose blot was stripped and reprobed with a β-actin probe to check for equal loading.

RT-PCR analysis was performed using cDNA templates from resting and activated CD4+ T cells, CD19+ B cells, CD8+ T cells, and resting CD14+ monocytes (CLONTECH Laboratories). The PCR reaction was performed in a 50-μl volume containing 5 μl of cDNA, 50 mM KCl, 10 mM Tris- HCl, pH 9.0, 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 mM dNTP, 5 pmol of each primer, and 1.25 units of Taq polymerase. The reaction mixture was denatured by heating at 94 °C for 30 s. Denaturation was followed by 30 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 5 min. The PCR products were analyzed by gel electrophoresis. Primers used for the RT-PCR were as follows: SLAP-2: ggaaggctgagaggtctagcaggg, ggaaggctgagaggtctagcaggg, s and ggaaggctgagaggtctagcaggg.

Cell Culture, Growth Factors, and Antibodies—Jurkat T cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and penicillin/streptomycin. Peptide corresponds to the COOH-terminal human SLAP-2 was synthesized by Boston Biomolecules (Woburn, MA). The specific SLAP-2 rabbit polyclonal antibody (anti-SLAP-2) was raised at Covance Research Products Inc. (Denver, PA). Anti-Shc, anti-SHP-2, and anti-Cbl antibodies were purchased from Transduction Laboratories (Lexington, KY), anti-V5 from Invitrogen, anti-phosphotyrosine (4G10) and anti-ZAP-70 from Upstate Biotechnology (Lake Placid, NY), and anti-CD3e from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Electroporation, Lipofection, and Luciferase Assays—For experiments in which IL-2 luciferase reporter was used, 2 × 106 Jurkat T cells were electroporated with 20 μg of reporter, 20 μg of each luciferase control and 0.2 ng of pCMV-β-galactosidase using a Bio-Rad electroporator. For experiments involving NF-AT luciferase reporter, 2 × 106 Jurkat T cells were transfected with 2 μg of reporter and 1.8 μg of different plasmid constructs using LipofectAMINE Plus (Invitrogen, Gaithersburg, MD). Cells were subsequently grown for an additional 16–18 h. The cells were then treated with 1 μg/ml anti-human CD3, cloneUCHT1 (PharMingen, Int., San Diego, CA) plus 5 μg/ml rabbit anti-mouse antibody (Dako, Denmark) or 50 ng/ml phorbol 12-myristate 13-acetate (PMA) plus 1 μM ionomycin (Sigma) for 8 h. After treatment, cells were harvested and luciferase and β-galactosidase activities were measured according to the manufacturer’s instructions (Tropix, Bedford, MA).

Immunoprecipitation and Western Blotting—For testing expression of endogenous SLAP-2, 7 × 106 Jurkat T cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1% SDS) with 1 mM sodium orthovanadate and protease inhibitors. Samples were incubated either with preimmune serum or SLAP-2 antisem. Immunoprecipitated proteins were resolved by SDS-PAGE. Membranes were incubated with SLAP-2 antibodies (anti-Myc, rabbit IgG (F(ab)′2) followed by horseradish peroxidase antibody for detection. In the phosphorylation assay, 3.5 × 106 Jurkat T cells were used per treatment. After stimulation for 5 min with anti-CD3 antibody (C305), cells were lysed in RIPA buffer and immunoprecipitated with anti-SLAP-2, anti-Shc, or anti-SHP-2 antibodies. Western blotting was performed with anti-phosphotyrosine antibody. Subsequently, the membranes were stripped by incubating the blot in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol) and reprobed with the respective primary antibodies. 293T cells were transfected using the calcium phosphate method with 15 μg of pEF/V5-His (Gateway™ modified) or vectors expressing various SLAP-2 V5-tagged constructs. Forty-eight hours after transfection, cells were lysed in “modified RIPA” (RIPA without SDS) with 1 mM sodium orthovanadate and incubated with anti-SLAP-2 antibodies. Membranes were incubated with 15 μg/ml anti-Myc, rabbit IgG, or anti-SHP-2 antibody. In GST pulldown experiments, 2.5 × 107 Jurkat T cells were lysed in lysis buffer and incubated for 4 h at room temperature with GST alone or GST-Cbl N. The Western blot was subsequently probed with anti-SLAP-2 antisem.

RESULTS AND DISCUSSION

Cloning of SLAP-2, a Novel SH2 and SH3 Domain Containing Adapter Protein—To identify novel signaling molecules, we screened EST data bases (dbEST) for sequences containing SH2 or SH3 domains. One of the mouse ESTs derived from a mouse embryo cDNA library identified in this search (IMAGE accession number 478854) contained a SH3 domain that was homologous to the Src family of tyrosine kinases. This clone was sequenced completely and the open reading frame was found to encode a protein of 259 amino acids. In addition to an SH3 domain, this cDNA also encodes an SH2 domain and was named cDNA clone SLAP-2. The absence of the 104 residue COOH-terminal domain that is different from other previously identified molecule called Src-like adapter protein (18) and therefore designated as SLAP-2. SLAP-2 contains unique SH2-terminal and COOH-terminal regions that are not homologous to any other protein in databases. Using BLAST search, we identified a human cDNA clone (GenBank™ accession number AK025645) isolated from a...
HepG2 hepatoma cell line that was labeled as encoding an unnamed protein product. Analysis of the protein encoded by the open reading frame contained in this clone revealed it to be the human ortholog of murine SLAP-2. Fig. 1A shows an alignment of murine and human SLAP-2 proteins that are 79% identical to each other. Both of them contain the sequence, FLIRES, which corresponds to a conserved motif located in the phosphotyrosine binding pocket of all SH2 domains (19). The strong conservation between human and murine SLAP-2 is also reflected in the genomic structure of the murine and human SLAP-2 coding regions obtained by comparison of the respective cDNAs to mouse and human genomic sequences. The coding sequence of murine and human SLAP-2 is distributed over 7 exons with a complete conservation of the length of the coding regions except for one extra amino acid in the first exon and 3 additional amino acids in the last coding exon of human SLAP-2 (Fig. 1B). The intron-exon boundaries are completely conserved and follow the GT/AG rule (20) (Tables I and II).

**mRNA Expression of SLAP-2**—To determine the tissue distribution of SLAP-2 mRNA, we probed two Northern blots containing poly(A)+ mRNA from different human tissues with a specific cDNA fragment from human SLAP-2. SLAP-2 expression was indicated by the presence of one major mRNA species of ~2.4 kb only in the tissues from the immune system with highest levels seen in peripheral blood leukocytes (Fig. 2A). We also took advantage of the presence of expressed sequence tags (ESTs) corresponding to SLAP-2 as an indicator of expression in various tissues, a so-called “electronic Northern” (21), by performing a BLAST search against the EST data base. We found EST entries corresponding to SLAP-2 from cDNA libraries generated from spleen, thymus, and lymph nodes but also from placenta, prostate, skin, retina, and colon indicating that SLAP-2 may also be expressed at low levels in other tissues. In addition, an EST that was derived from Jurkat T cell line was found in this search. To further characterize which cell populations of the immune system express SLAP-2 mRNA, we performed RT-PCR in resting and activated CD4+ and CD8+ T cells, in resting and activated CD19+ B cells and resting CD14+ monocytes (Fig. 2B). We were able to detect SLAP-2 transcript in all the samples analyzed; however, the levels in resting and activated CD19+ B cells were very low compared with that observed in the rest of the samples analyzed. Therefore, although SLAP-2 is expressed in T and B cells as well as monocytes, its expression is low in B cells.

**Involvement of SLAP-2 in TCR Signaling**—Since SLAP-2...
mRNA was expressed in T cells, it is possible that it plays a role in T cell signaling. To check expression of SLAP-2 protein, we generated a polyclonal antibody directed against a peptide derived from the COOH terminus of SLAP-2. Immunoprecipitation and Western blotting of Jurkat T cell lysates with anti-SLAP-2 antiserum revealed the presence of two bands with molecular weights of 27,000 and 25,000. These bands were specific since they were not immunoprecipitated by the preimmune serum. The size of the upper band is close to the predicted molecular weight of 28,000 deduced from the open reading frame of SLAP-2 (Fig. 3A). The origin of the lower band is not clear although it may represent a processed form of SLAP-2, a degradation product or may arise from an internal translation initiation site in its mRNA.

TCR Activation Does Not Induce Tyrosine Phosphorylation of SLAP-2—Cross-linking of TCR leads to activation of several tyrosine kinases that in turn phosphorylate other proteins thereby initiating a signaling cascade. Several of these downstream proteins such as LAT, Cbl, SHP-2, and Shc undergo tyrosine phosphorylation in response to TCR activation (22–25). To test if SLAP-2 is similarly phosphorylated on tyrosine residues upon TCR activation, Jurkat T cells were stimulated with an anti-CD3 antibody. Unstimulated and stimulated lysates were immunoprecipitated with anti-SLAP-2 antiserum followed by Western blotting with anti-phosphotyrosine antibody. In parallel, as controls for TCR activation, the samples were immunoprecipitated with antibodies specific for Shc, an adapter protein, and SHP-2, a cytoplasmic phosphatase. Although we could easily detect an increase in tyrosine phosphorylation of Shc and SHP-2, there was no detectable phosphorylation of SLAP-2 (Fig. 3B). Reprobing of the membranes with the corresponding specific antibodies showed that equal amounts of SLAP-2, Shc, and SHP-2 proteins were loaded. These findings suggest that tyrosine phosphorylation may not be required for the function of SLAP-2.

Inhibition of TCR Signaling by SLAP-2—To study the function of SLAP-2, we cloned the open reading frame of SLAP-2 into a mammalian expression vector which provides a V5 epitope tag at the COOH terminus. Expression of epitope-tagged SLAP-2 was confirmed by transfection of the plasmid

**Table I**

Intron-exon boundaries of the marine SLAP-2 gene

The coding exon numbers are shown in the left column of the table. The amino acids are shown above the corresponding nucleotide sequences and the length of the introns is indicated.

| No | Exon | 5’ splice donor | Intronic size (bp) | 3’ splice acceptor | Exon |
|----|------|-----------------|-------------------|-------------------|------|
| I  | P    | gatagt          | 3378              | ctctag            | R R  |
| II | E    | gatagt          | 118               | cccacag           | G    |
| III|    | gatagt          | 675               | ctctag            | W L  |
| IV |    | gatagt          | 2733              | cccacag           | G C Y|
| V  |    | gatagt          | 813               | cccacag           | A C G|
| VI |    | gatagt          | 340               | cccacag           | C A C|

**Table II**

Intron-exon boundaries of the human SLAP-2 gene

The coding exon numbers are shown in the left column of the table. The amino acids are shown above the corresponding nucleotide sequences and the length of the introns is indicated.

| No | Exon | 5’ splice donor | Intronic size (bp) | 3’ splice acceptor | Exon |
|----|------|-----------------|-------------------|-------------------|------|
| I  | A    | gatagt          | 6666              | ccttag            | R S  |
| II | E    | gatagt          | 861               | cccacag           | D     |
| III|    | gatagt          | 845               | cccacag           | W L  |
| IV | K    | gatagt          | 17221             | ccttag            | G S Y|
| V  | Y    | gatagt          | 768               | cccacag           | E L A|
| VI |    | gatagt          | 319               | cccacag           | S L  |

**Fig. 2.** Tissue distribution of SLAP-2 mRNA and expression in purified human blood cell fractions. A, Northern blot analysis of SLAP-2 in various tissues. All lanes contain 2 μg of immobilized poly(A) RNA. The sizes of the transcripts are indicated in kb on the left. The top panel shows the results of probing the blot with a 32P-labeled fragment derived from the coding region of human SLAP-2. Equal loading was confirmed by the reprobing the blot with a β-actin cDNA probe as shown in the lower panels. B, RT-PCR was performed using primers specific for human SLAP-2 on cDNAs obtained by reverse transcription of mRNAs from the indicated purified cell populations. The bottom panel shows the amplification of a fragment specific for the glycerol-3-phosphate dehydrogenase (G3PDH) used as a control for equal amount of cDNA.
SLAP-2 as a Negative Regulator of T Cell Receptor Signaling

The production of IL-2 upon activation of TCR by anti-CD3 can be mimicked by a simultaneous increase of calcium levels and the activation of protein kinase C by using a combination of PMA and ionomycin (28, 29). To further study the effects of SLAP-2, we examined the activity of NF-AT luciferase and IL-2 luciferase after treating cells with PMA and ionomycin. It was found that the overexpression of SLAP-2 had no inhibitory effect on the luciferase activity of IL-2 or NF-AT constructs (Fig. 4B). This observation indicates that the negative regulation of SLAP-2 is upstream of an increase in calcium and Ras activation.

Requirement of an Intact SH2 Domain for the Inhibitory Effect of SLAP-2—To delineate the region of SLAP-2 that is responsible for the negative regulation of TCR signaling, we generated point mutants that inactivated the SH3 or the SH2 domain of SLAP-2. To disrupt the tyrosine binding capability of its SH2 domain, we mutated the arginine residue within the phosphotyrosine binding pocket to glutamic acid (5, 30, 31). SH3 domains contain a conserved proline residue that is critical for peptide binding (32–34) and, therefore, we mutated the equivalent proline at position 82 in its SH2 domain. To disrupt the tyrosine binding capability of SLAP-2 to a leucine residue. The expression of both of these mutants was found to be similar to that of wild type SLAP-2 (Fig. 3G). When Jurkat T cells were co-transfected with the SH2 mutant of SLAP-2, no inhibition of NF-AT luciferase activity was observed upon cross-linking the TCR. In contrast, when cells were co-transfected with the SH3 mutant of

![Figure 3.](http://www.jbc.org/)

**Endogenous SLAP-2 does not get tyrosine phosphorylated upon TCR activation.** A, endogenous expression of SLAP-2 in Jurkat T cell line. Jurkat T cell lysates were immunoprecipitated with preimmune serum or immune antiserum generated against human SLAP-2 as indicated. Western blotting with SLAP-2 antisera shows two specific bands (indicated by arrows). The molecular mass markers in kDa are indicated on the right. B, SLAP-2 does not get tyrosine phosphorylated upon TCR activation. Jurkat T cells were left untreated (−) or incubated for 5 min with anti-CD3 antibody (+). The upper panel shows Western blotting with anti-phosphotyrosine antibody of samples immunoprecipitated with anti-SLAP-2, anti-Shc, or anti-SHP-2 as indicated. The blot for SLAP-2 and SHP-2 were reprobed with the same antibody to ensure similar loading. C, expression of various SLAP-2 constructs. 293T cells were transfected with various V5 epitope-tagged constructs as indicated and expression of proteins detected by immunoprecipitation of cell lysates followed by Western blotting using anti-V5 antibody.

![Figure 4.](http://www.jbc.org/)

**Inhibition of TCR signaling by SLAP-2.** A, Jurkat T cells were transfected with NF-AT luciferase (left) or IL-2 luciferase (right) constructs, together with empty vector or SLAP-2 as described under “Experimental Procedures.” Approximately 16–18 h after transfection, half the cells were incubated for an additional 8 h with anti-CD3 antibody (+) and the other half left untreated (−). Subsequently, the luciferase activity was measured. B, Jurkat T cells were transfected with NF-AT luciferase (left) or IL-2 luciferase (right) constructs as in panel A, together with empty vector or SLAP-2. The procedures were as in panel A except that a combination of PMA and ionomycin was used for stimulation. All the experiments were repeated at least three times with similar results; a representative experiment is shown.
SLAP-2, a reduction of luciferase activity in stimulated cells was similar to that seen with transfected wild type SLAP-2 was observed (Fig. 5A). Therefore, the SH2, but not the SH3 domain, is necessary for the attenuation of TCR signaling by SLAP-2. These observations suggest that the binding of SH2 domain of SLAP-2 to tyrosine-phosphorylated proteins in the TCR signaling pathway may be essential for its inhibitory function. Since the SH2 domain of SLAP-2 is homologous to the Src family of kinases, one of the mechanisms of inhibition may be that it competes with Lck to inhibit TCR signaling. Indeed, SLAP-2 has previously been shown to inhibit platelet-derived growth factor-induced mitogenesis in NIH 3T3 fibroblasts and to compete with c-Src for binding to the same sites on the platelet-derived growth factor receptor (35).

Inhibition by SLAP-2 Is Suppressed by Disruption of the Predicted Myristoylation Site—Targeting of proteins into specialized membrane subdomains is a potential mechanism to organize and facilitate the assembly of the signaling cascade by bringing protein effectors in proximity to their substrates. For instance, exclusion of the adapter LAT from specialized membrane subdomains leads to impaired TCR signaling (36). Several members of the Src family are known to be associated with the plasma membrane by means of two fatty acyl chains: myristic acid attached to a glycine residue and palmitic acid attached to a cysteine, both located within the NH2-terminal region (37, 38). The NH2 terminus of SLAP has also been reported to be myristoylated at a glycine located at position 2 and to co-localize with Src in vivo (39). Mutation of this glycine residue to alanine is expected to prevent lipid attachment leading to changes in the localization of SLAP (39). Both murine and human SLAP-2 sequences shown conservation of a consensus motif, MGXXXS, for myristoylation at their NH2 termini. We therefore decided to test if the conserved glycine residue was necessary for the observed SLAP-2 inhibitory effect by mutating the glycine at the position 2 into alanine. Expression of SLAP-2 G2A mutant was first confirmed by Western blotting with anti-V5 antibody (Fig. 3C). When Jurkat T cells were transfected with this mutant together with NF-AT luciferase, we did not observe any inhibition of the stimulation upon anti-CD3 cross-linking (Fig. 5B). This is analogous to the decrease in activation of Lck that is observed when the corresponding glycine residue in Lck is mutated to an alanine (41). These results suggest localization of SLAP-2 near the plasma membrane may indeed be required for its inhibitory role in TCR signaling.

SLAP-2 Associates with Several Phosphoproteins Involved in TCR Signaling.—Since the SH2 domain of SLAP-2 is critical for the inhibition of TCR signaling we sought to identify tyrosine-phosphorylated proteins that interact with SLAP-2. For this purpose, lysates from untreated and stimulated Jurkat T cells were immunoprecipitated with SLAP-2 antiserum and probed with anti-phosphotyrosine antibody. We found that SLAP-2 interacted with phosphorylated ZAP-70 and CD3 \( \zeta \) chain (Fig. 6A). These proteins have also been previously shown to interact in a GST pulldown with the prototypical member of this family, SLAP (17, 27).
CONCLUSIONS

Our results indicate that SLAP-2 can negatively regulate TCR signaling. SLAP-2 is proximal to the activation of TCR as its inhibitory effect cannot be observed after a simultaneous increase of calcium levels and Ras activation by addition of PMA plus ionomycin. The inhibition of TCR signaling pathway requires an intact SH2 domain whereas the SH3 domain is not critical for this effect. The NH₂ terminus of SLAP-2 is most likely myristoylated and the integrity of the predicted myristoylation site is essential for the observed down-regulation of TCR signaling. Since both SLAP and SLAP-2 are coexpressed in thymus, spleen, and lymph node, it is possible that they have at least partially redundant roles in T cell signaling. This notion is supported by the fact that they share common binding partners such as ZAP-70, CD3ζ, and Cbl. The negative regulation by SLAP was confirmed by an enhanced positive selection of thymocytes that was observed in SLAP knockout mice (43). In addition, absence of SLAP was able to partially rescue T cell development in mice that were deficient in ZAP-70 (43).

The COOH-terminal region of SLAP has been shown to interact with Cbl although the exact residues responsible for this interaction have not been delineated (17). The C terminus of SLAP-2 is not very similar to that of SLAP and it lacks the last 27 amino acids found in SLAP that contain highly charged residues. We have not been able to detect the presence of any other adapter protein with the same modular arrangement in the human genome, therefore SLAP and SLAP-2 represent a family of proteins that may have partially overlapping functions. It is striking that although the SH2 domains of murine and human SLAP-2 and SLAP are 60% identical, the SH3 domains are only 37–45% identical. Identification of the binding partners of SLAP-2 in cells will not only help in elucidation of its exact mechanism of action but also explain if there are any differential effects as compared with SLAP. While this article was under review, another report describing identification of SLAP-2 was published (44). Holland et al. (44) cloned SLAP-2 by a functional strategy designed to isolate inhibitors of the B cell receptor signaling pathway. Our results are in keeping with their findings that show that overexpression of SLAP-2 inhibits TCR signaling and that mutation of the conserved myristoylation sequence is required for this inhibitory function (44).

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Fig. 7. SLAP-2 interacts in vitro and in vivo with Cbl. A, 293T cells were transfected with vector or SLAP-2 V5-tagged construct, and hemagglutinin (HA)-tagged Cbl construct as indicated. Anti-HA antibody was used for Western blotting of the immunoprecipitated lysates to visualize co-precipitating Cbl. B, lysates from Jurkat T cells were incubated with the GST constructs indicated. The top panel shows the Western blot using anti-SLAP-2 antiserum to detect SLAP-2 bound to the indicated GST fusion proteins. Two specific bands corresponding to SLAP-2 are indicated. The bottom panel shows Amido Black staining of the membrane with the arrows indicating the position of the GST fusion proteins used in the pulldown. C, in a similar experiment to the one described in Fig. 6A, Western blotting was performed with anti-Cbl antibody to detect Cbl bound to SLAP-2 in unstimulated and anti-CD3 stimulated Jurkat T cells.
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