Communication

Molecular Cloning of SLAP-130, an SLP-76-associated Substrate of the T Cell Antigen Receptor-stimulated Protein Tyrosine Kinases*

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Previous work has demonstrated that SLP-76, a Grb2-associated tyrosine-phosphorylated protein, augments interleukin-2 promoter activity when overexpressed in the Jurkat T cell line. This activity requires regions of SLP-76 that mediate protein-protein interactions with other molecules in T cells, suggesting that SLP-76-associated proteins also function to regulate signal transduction. Here we describe the molecular cloning of SLAP-130, a SLP-76-associated phosphoprotein of 130 kDa. We demonstrate that SLAP-130 is hematopoietic cell-specific and associates with the SH2 domain of SLP-76. Additionally, we show that SLAP-130 is a substrate of the T cell antigen receptor-induced protein tyrosine kinases. Interestingly, we find that in contrast to SLP-76, overexpression of SLAP-130 diminishes T cell antigen receptor-induced activation of the interleukin-2 promoter in Jurkat T cells and interferes with the augmentation of interleukin-2 promoter activity seen when SLP-76 is overexpressed in these cells. These data suggest that SLP-76 recruits a negative regulator, SLAP-130, as well as positive regulators of signal transduction in T cells.

Engagement of the T cell antigen receptor (TCR) results in the activation of protein tyrosine kinases (PTK) and the subsequent tyrosine phosphorylation of numerous proteins in T cells (1). Our efforts to characterize substrates of the TCR-induced PTK activity led to the cloning of SLP-76, a tyrosine-phosphorylated hematopoietic cell-specific protein that associates with the SH3 domains of Grb2 (2, 3). A possible function of SLP-76 in T cells was suggested by experiments showing that overexpression of SLP-76 augments TCR-mediated signals that lead to the induction of IL-2 gene promoter activity (4, 5). We have shown that the activity of SLP-76 requires engagement of the TCR and that overexpression of SLP-76 results in increased activation of the mitogen-activated protein kinase cascade following TCR ligation (2). Interestingly, three distinct regions of SLP-76 that are responsible for protein-protein interactions in T cells are required for its ability to augment IL-2 promoter activity when overexpressed (6, 7). These data suggest that SLP-76 functions as a link between proteins that regulate signals generated by TCR ligation.

To investigate the function of SLP-76 in T cells further, we and others have begun to characterize SLP-76-associated proteins that may participate with SLP-76 in transducing signals from the TCR to the nucleus. These proteins include Vav, which associates with the amino-terminal acidic region of SLP-76 in a phosphotyrosine-dependent manner (5, 8, 9); the adapter protein Grb2, which interacts with a proline-rich motif of SLP-76 via its SH3 domains (3, 4); and two unidentified tyrosine-phosphorylated proteins of 64 and 130 kDa and a serine/threonine kinase, all of which associate with the carboxyl-terminal SH2 domain of SLP-76 (4). In this study, we report the cloning of the cDNA encoding a 130-kDa protein (SLAP-130 for SLP-76 associated phosphoprotein of 130 kDa) that associates with the SH2 domain of SLP-76. Additionally, we provide evidence that SLAP-130 may function as a negative regulator of TCR signals that activate IL-2 gene transcription.

MATERIALS AND METHODS

Cells and Cell Culture—Jurkat T cells were maintained as described (10). JA2/SLP-SH2, a Jurkat T cell variant expressing an A2/SLP-SH2 chimera, was maintained in medium supplemented with 2 mg/ml gentamicin (Life Technologies, Inc.).

cDNA Constructs—The SH2 domain of SLP-76 was amplified by PCR and subcloned in frame with HLA-A2 in pcDNA3/A2/HCP (10) to generate pcDNA3/A2/SLP-SH2. The amino-terminal 1350 nucleotides of SLP-130 were amplified from Jurkat RNA and ligated in frame with the FLAG tag in pEF/SLP-SH2 (4). The remaining 3′ cDNA was amplified from Jurkat RNA by overlap extension PCR. The resulting fragment was ligated in frame with the amino-terminal 1350 bp in pEF above to create pEF/SLAP-130. pEF/A2, the expression vector containing HLA-A2 cDNA, was a gift of B. Shraven (University of Heidelberg, Germany). NFAT-luc was a gift of G. Crabtree (Stanford University, Palo Alto, CA). The expressed sequence-tagged (EST) clone (I.M.A.G.E. consortium ID number 241254) was obtained from Genome Systems, Inc. (St. Louis, MO).

Antibodies—Anti-A2 mAb CR11–351 (gift of C. Lutz, University of Iowa, Iowa City, IA). Anti-TCR mAb C305 (gift of A. Weiss, University of Iowa, Iowa City, IA). The expressed sequence-tagged (EST) clone (I.M.A.G.E. consortium ID number 241254) was obtained from Genome Systems, Inc. (St. Louis, MO).

The abbreviations used are: TCR, T cell antigen receptor; PTK, protein tyrosine kinase; NFAT, nuclear factor of activated T cells; IL, interleukin; bp, base pair(s); mAb, monoclonal antibody; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; EST, expressed sequence-tagged; PAGE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction.

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2 M. A. Musci, D. G. Motto, S. E. Ross, N. Fang, and G. A. Koretzky, submitted for publication.
of California, San Francisco, CA). Anti-FLAG mAb M2 (International Biotechnologies Inc., New Haven, CT). Anti-phosphotyrosine mAb 4G10 (Upstate Biotechnologies Inc., Lake Placid, NY). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-sheep antiserum (Bio-Rad). Anti-SLP-76 sheep antiserum has been described previously (14). An anti-A2 monoclonal antibody was generated against a GST fusion protein containing amino acids 1–340 of human SLP-130.

Immunoprecipitations—Cells were left unstimulated or stimulated with anti-TCR mAb (C305, acites, 1:1000) for the indicated times or pervanadate for 2 h at 4 °C. Lysates were subjected to precipitation with the indicated antibodies or GST fusion protein for 2 h at 4 °C. Precipitated complexes were washed four times in high salt lysis buffer (500 mM NaCl), resolved by reducing SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and visualized by Ponceau S staining. The protein band of 130 kDa was excised and subjected to trypsin digestion and reverse phase high performance liquid chromatography for protein sequencing.

Individual peptides were sequenced using a Procise 492 protein sequencer (Perkin-Elmer/AABD, Foster City, CA). One peptide sequence, PPNDVLTK, was represented in an EST clone containing an open reading frame of 1074 bp. Nucleotides 1088–1296 (thereby amplified by PCR from the EST and used to screen a human thymus gt10 cDNA library (#NL1127a, Promega, Madison, WI). A λ clone was identified containing 1008 bp 5′ of the EST. The remaining cDNA was amplified from Jurkat cDNA by 3′ RACE (rapid amplification of cDNA ends) using the Marathon cDNA Amplification Kit (CLONTECH, Palo Alto, CA). Independent amplification of the entire coding sequence of SLP-130 from Jurkat RNA was performed by RT-PCR using the GeneAmp kit (Perkin-Elmer).

Northern Analysis—Northern blot analysis was performed following the protocols included with the human multiple tissue Northern Blots I and II (CLONTECH). A SLP-130 PCR product (nucleotides 1088–1296) was labeled with [α-32P]dCTP (Amersham) by random priming and hybridized to poly(A)− RNA from multiple tissues. Transfections and NTA Luciferase Assays—Cells were washed in phosphate-buffered saline and suspended in cytomyxin (12) at a concentration of 2 × 106 cells/400 μl of cytomyxin/cuvette. Cells were electrophoresed at 250V, 960 mA using a Gene Pulser (Bio-Rad) with 15 μg of NTA-luc, 5 μg of cytomegalovirus-β-galactosidase, and 40 μg of either pEFSL−76 or pEFSL−130. The total amount of plasmid DNA was equimolar to 100 μg with the vector control pEF/A2. After 24 h, 5 μl of 107 cells were stimulated in triplicate for 10 h with media or anti-TCR mAb C305 (acites 1:1000). Additionally, triplicate samples of 5 × 106 unstimulated cells were assayed for β-galactosidase activity using the Galacto-Light Plus Reporter Gene Assay System (Tropix Inc., Bedford, MA). Luciferase activity was determined as described previously (10). Luciferase light units were normalized to β-galactosidase activity present in each transfectant to standardize for transfection efficiency.

RESULTS AND DISCUSSION

Cloning of cDNA Encoding SLP−130—We have shown previously that the SH2 domain of SLP-76 associates with two unidentified tyrosine-phosphorylated proteins upon stimulation of Jurkat T cells with either anti-TCR mAb or the protein tyrosine phosphatase inhibitor pervanadate (4). To facilitate purification of these SLP-76-associated proteins, we established a variant of the Jurkat T cell line, JA2/SLP-SH2, which expresses a chimeric surface protein consisting of the extracellular and transmembrane domains of the HLA-A2 molecule in frame with the SH2 domain of SLP-76. The A2 epitope enabled the isolation of proteins associated with the SH2 domain of SLP-76 by immunoprecipitation with anti-A2 mAb.

JA2/SLP-SH2 cells were stimulated with pervanadate for maximal tyrosine phosphorylation of proteins and lysed in Nonidet P-40 lysis buffer. Anti-A2 immunoprecipitates were resolved on SDS-PAGE and transferred to polyvinylidene difluoride membrane. A single major species of approximately 130 kDa was excised and subjected to trypsin digestion to generate peptides for protein sequencing. One peptide sequence, PPNDVLTK, was represented in an EST clone. Sequencing this clone revealed an open reading frame of over 1000 bp. A region of this clone was then used to probe a human thymus gt10 cDNA library to obtain further sequence.

A cDNA clone containing 370 bp of the EST sequence and an additional 1008 bp of coding sequence 5′ of the EST was isolated and found to contain a putative start site 27 bases downstream of a stop codon. A cDNA containing the remaining 3′-coding sequence was amplified from Jurkat cDNA by 3′ RACE and contained 267 additional bases of coding sequence followed by a stop codon 2349 bases downstream of the putative start. A contiguous cDNA was generated by RT-PCR amplification of pp130 from Jurkat RNA (Fig. 1). This sequence was confirmed by sequencing the product of multiple independent RT-PCR reactions. We have designated this protein SLP-130 for SLP-76 associated phosphoprotein of 130 kDa.

The complete open reading frame of SLP-130 consists of 2349 bp translating to a protein of 783 amino acids with an abundance of proline (12%) and charged (31%) residues. Putative nuclear localization sequences are found within amino acids 480–503 and 683–700 (Ref. 13 and see Fig. 1). We do not know yet whether SLP-130 is present in the nucleus of Jurkat T cells. There are several potential tyrosine phosphorylation sites in the carboxyl-terminal region of the molecule that may be responsible for the interaction of SLP-130 with the SH2 domain of SLP-76. In addition, there are four tyrosines (residues 462, 595, 651, and 771) present in motifs that may mediate binding to the SH2 domain of src family kinases (14), suggesting that SLP-130 may interact with proteins other than SLP-76 in T cells.

Tissue Distribution of SLP-130—Northern blot analysis of human mRNA from multiple tissues was performed to determine the tissue distribution of SLP-130 (Fig. 2). As shown, SLP-130 mRNA is expressed in hematopoietic tissues (peripheral blood mononuclear cells, spleen, and thymus) but not in non-hematopoied tissues. Additionally, SLP-130 was amplified as a single product from total Jurkat RNA (data not shown), demonstrating expression in this cell line.

Expression of cDNA Encoding SLP-130 Results in a Protein That Migrates Similarly to pp130—Translation of the open reading frame of the SLP-130 cDNA predicts a protein with a molecular mass of 86 kDa. However, the presence of stop codons flanking both the 5′ and 3′ ends of the putative coding sequence suggested that we had isolated the full-length cDNA encoding a 130-kDa, SLP-76-associated protein. This was confirmed by generating an epitope (FLAG)-tagged version of SLP-130 cDNA (pEF-SLAP-130) for transient expression in Jurkat T cells. As shown in Fig. 3A, transfection of Jurkat T cells with pEF-SLAP-130 results in the expression of a protein reactive with anti-FLAG mAb that migrates with an apparent molecular mass of 130 kDa (lane 2). This protein does not appear in lysates of cells transfected with the control vector pEF (lane 1). Whether the apparent migration of SLP-130 at 130 kDa is consistent with the predicted 86 kDa results from posttranslational modifications or the abundance of charged amino acids present in the molecule is not yet known.

To investigate further whether the protein encoded by the SLP-130 cDNA is identical to the 130-kDa, SLP-76-associated phosphoprotein, we prepared a GST fusion protein containing the SH2 domain of SLP-76 (GST/SH2). As shown in Fig. 3B, this fusion protein precipitates epitope-tagged SLP-130 from pervanadate-stimulated Jurkat cells (lane 4), but not from resting cells (lane 3) transfected with pEF-SLAP-130. The loss of function mutant of the SLP-76 SH2 domain when expressed as a GST fusion protein (GST/R446K) fails to associate with FLAG-SLAP-130 in either resting or pervanadate-stimulated

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cells (lanes 1 and 2). The protein precipitated by the wild type SLP-76 SH2 domain migrates identically to a protein detected by anti-FLAG immunoblot analysis of an anti-FLAG immunoprecipitate (lane 5) or whole cell lysates (lane 6) from Jurkat cells transfected with pEF/SLAP-130. Additionally, the FLAG-SLAP-130 protein migrates identically to an endogenous tyrosine-phosphorylated protein precipitated by the wild type SH2 domain of SLP-76 (lanes 1, 3, 5, and 6). The amount of SLAP-130 precipitated by the wild type SH2 domain of SLP-76 was found to increase following stimulation of Jurkat cells with pervanadate (lane 7). We next investigated whether SLAP-130 is a substrate of the TCR-induced PTKs. Jurkat T cells were left unstimulated or stimulated with anti-TCR mAb or pervanadate. At the indicated time points whole cell lysates were subjected to immunoblot analysis with anti-FLAG mAb. As noted above, there is a basal level of tyrosine phosphorylation of SLAP-130, and this level increases following stimulation of Jurkat cells with pervanadate (lane 5).

We and others have shown that overexpression of SLAP-130 interferes with TCR-mediated signal transduction (4, 7). We next determined if SLAP-130 associates with SLP-76 in resting Jurkat cells. To this end, we transfected Jurkat with a retroviral expression vector that encodes a FLAG-tagged SLAP-130 from stimulated Jurkat T cells. Jurkat T cells transiently transfected with pEF/SLAP-130 contain a 130-kDa protein reactive with anti-FLAG mAb. As shown in Fig. 3A, lane 7, this 130-kDa protein precipitated by the wild type SH2 domain of SLP-76 is a tyrosine-phosphorylated protein that is not precipitated by an analogous fusion protein containing a loss of function SLP-76 SH2 domain (lane 2). Additionally, anti-phosphotyrosine (a-PY) Western blot of a GST/SHP2 precipitate from untransformed pervanadate-stimulated Jurkat cells demonstrates that the 130-kDa protein associated with the SH2 domain of SLP-76 migrates the same as SLAP-130 (lane 7).

In the resting state, as shown in Fig. 3B, the amount of SLAP-130 associated with SLP-76 increases following stimulation of Jurkat with pervanadate (lane 5).
SLAP-130 associates with SLP-76 in Jurkat T cells and is a substrate of the TCR-induced PTKs. A. Anti-SLAP-130 antiserum. 3 × 10⁷ Jurkat T cells were subjected to immunoprecipitation with either preimmune serum (lane 1) or anti-SLAP-130 antiserum (lane 2). These immune complexes, in addition to whole cell lysates (lane 3), were subjected to Western blot analysis with anti-SLAP-130 antiserum. Whole cell lysates prepared from 5 × 10⁷ unstimulated (lane 4) or pervanadate-stimulated (lane 5) Jurkat T cells were subjected to immunoprecipitation with anti-SLP-76 antiserum and then immunoblotted with anti-SLP-76 (bottom panel) and anti-SLAP-130 (top panel) antisera. B, tyrosine phosphorylation of SLAP-130 in Jurkat T cells. 2 × 10⁶ Jurkat T cells were left unstimulated or stimulated with anti-TCR mAb (C305 actes 1:1000) for indicated times or pervanadate for 1 min. Whole cell lysates were subjected to immunoprecipitation with anti-SLAP-130 antiserum and Western blot analysis with anti-phosphotyrosine mAb (a-PY, top panel). The a-PY blot was stripped and immunoblotted with anti-SLAP-130 antiserum (lower panel) to demonstrate the amount of SLAP-130 in each lane.

Regulate signals through the TCR in a positive manner. To determine the effect of SLAP-130 on signals generated by TCR ligation, Jurkat cells were transiently transfected with pEF-SLAP-130 or a control vector and a luciferase reporter construct driven by the NFAT response element. Fig. 4A demonstrates that, in contrast to the effect of SLP-76 on T cell signaling, overexpression of SLAP-130 results in diminished NFAT activity following TCR ligation. Furthermore, co-transfection of SLAP-130 and SLP-76 reveals that overexpression of SLAP-130 inhibits the augmentation of TCR-stimulated IL-2 promoter activity by SLP-76. Expression of the FLAG-tagged cDNAs was confirmed by immunoblotting whole cell lysates with anti-FLAG mAb.

Northern blot analyses indicate that SLP-76 and SLAP-130 are expressed coordinately in hematopoietic tissues. Defining their role in other hematopoietic cells may provide insight into how these proteins regulate T cell signals. We have found an association between the SLP-76 SH2 domain and a 130-kDa protein in rat basophilic leukemia cells (15). Since engagement of the high affinity receptor for IgE on rat basophilic leukemia cells leads to tyrosine phosphorylation of SLP-76 (15), experiments are ongoing to determine if overexpression of SLP-76 augments signaling events downstream of receptor binding in these cells. Similarly, it will be of interest to determine if overexpression of SLAP-130 impacts negatively on signal transduction via the IgE receptor.

It will be important to determine if the down-regulation of TCR-induced NFAT activation by overexpression of SLAP-130 requires an interaction between SLP-76 and SLAP-130. Experiments are in progress to determine the site on SLAP-130 responsible for its interaction with SLP-76. Manipulation of the SLAP-130 cDNA prior to transfection into Jurkat cells expressing wild type or overexpressed levels of SLP-76 will facilitate our understanding of how these two molecules may interact to regulate signals downstream of TCR engagement.

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**FIG. 4.** SLAP-130 associates with SLP-76 in Jurkat T cells and is a substrate of the TCR-induced PTKs. A. Anti-SLAP-130 antiserum. 3 × 10⁷ Jurkat T cells were subjected to immunoprecipitation with either preimmune serum (lane 1) or anti-SLAP-130 antiserum (lane 2). These immune complexes, in addition to whole cell lysates (lane 3), were subjected to Western blot analysis with anti-SLAP-130 antiserum. Whole cell lysates prepared from 5 × 10⁷ unstimulated (lane 4) or pervanadate-stimulated (lane 5) Jurkat T cells were subjected to immunoprecipitation with anti-SLP-76 antiserum and then immunoblotted with anti-SLP-76 (bottom panel) and anti-SLAP-130 (top panel) antisera. B, tyrosine phosphorylation of SLAP-130 in Jurkat T cells. 2 × 10⁶ Jurkat T cells were left unstimulated or stimulated with anti-TCR mAb (C305 actes 1:1000) for indicated times or pervanadate for 1 min. Whole cell lysates were subjected to immunoprecipitation with anti-SLAP-130 antiserum and Western blot analysis with anti-phosphotyrosine mAb (a-PY, top panel). The a-PY blot was stripped and immunoblotted with anti-SLAP-130 antiserum (lower panel) to demonstrate the amount of SLAP-130 in each lane.

**FIG. 5.** Overexpression of SLAP-130 diminishes transcriptional activation through the NFAT response element. Jurkat T cells were transfected with NFAT-luc, cytomegalovirus-β-galactosidase, and either vector control, pEF-SLP-76, pEF/SLAP-130, or both pEF/SLAP-130 and pEF/SPLP-76. The amount of DNA in each sample was equalized with vector control. 24 h following transfection, cells were stimulated in triplicate with anti-TCR mAb for 10 h and then assayed for luciferase activity. Luciferase light units were normalized to β-galactosidase activity in each transfectant. Expression of the epitope-tagged constructs was determined by preparing whole cell lysates from 10⁷ transfected cells and immunoblotting with anti-FLAG mAb.
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