Implication of the GRB2-associated Phosphoprotein SLP-76 in T Cell Receptor–Mediated Interleukin 2 Production

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Summary

Recently we described the molecular cloning of SLP-76, a hematopoietic cell–specific 76-kD protein that was first identified through its association with GST/Grb2 fusion proteins. The primary sequence of SLP-76 predicts a protein of 533 amino acids comprising an amino-terminal region with numerous potential tyrosine phosphorylation sites, a central region rich in proline residues, and a single carboxy-terminal SH2 domain. Here we demonstrate formally that Grb2 associates with unphosphorylated SLP-76 and map the Grb2 binding site on SLP-76 to amino acids 224–244. We also demonstrate that upon T cell receptor (TCR) stimulation, SLP-76 undergoes rapid tyrosine phosphorylation and associates with tyrosine phosphoproteins of 36, 62, and 130 kD. In vitro experiments show that the SH2 domain of SLP-76 associates with the 62- and 130-kD proteins and additionally with a serine/threonine kinase. Finally, we demonstrate that transient overexpression of SLP-76 results in dramatically enhanced TCR-mediated induction of nuclear factor of activated T cells (NFAT) and interleukin (IL) 2 promoter activity; and we provide evidence that a functional SLP-76 SH2 domain is required for this effect. Our data document the in vivo associations of SLP-76 with several proteins that potentially participate in T cell activation and implicate SLP-76 itself as an important molecule in TCR-mediated IL-2 production.

L igation of the TCR results in the rapid activation of several protein tyrosine kinases (PTKs) with the subsequent phosphorylation of numerous cellular proteins (1–3), including components of the TCR itself (2, 3), Src and Syk family PTKs (Lck, Fyn, and ZAP-70 [for review see reference 4]), phospholipase Cγ1 (5–7), GAP (8), ezrin (9), CD5 (10, 11), Shc (12), Vav (13, 14), Cbl (15), and several cytoskeletal proteins (16). However, many substrates of the TCR-activated PTKs remain unidentified. Our laboratory has been interested in characterizing some of these molecules. As one strategy, we chose to investigate tyrosine phosphoproteins that associate with Grb2, an adapter molecule that has been shown to play a central role in coupling signaling pathways in other systems.

Using this approach, we and others have demonstrated that tyrosine phosphoproteins of 36, 76, and 116 kD associate with Grb2 in T cells (17–20). The 116-kD protein has been identified recently as the product of the c-Cbl protooncogene (15); however, its function remains unclear. The molecular identity of the 36-kD protein remains uncertain, but recent data from our laboratory suggest that tyrosine phosphorylation of this molecule is required for

coupling the TCR with the phosphatidylinositol second messenger pathway (Motto, D., M. Musci, S. Ross, and G. Koretzky, manuscript submitted for publication). Recently, in a collaborative effort (Paul Findell, Palo Alto, CA), we purified the 76-kD Grb2-associated phosphoprotein and cloned its cDNA, which encodes a novel hematopoietic cell-specific protein we have termed SLP-76, for SH2 domain leukocyte protein of 76 kD (21). The primary sequence of SLP-76 predicts a protein of 533 amino acids comprising an amino-terminal region with numerous potential tyrosine phosphorylation sites, a central region rich in proline residues, and a single carboxy-terminal SH2 domain. Using glutathione-S-transferase (GST)/SLP-76 fusion proteins and deletion analysis, we now have mapped the Grb2 binding site on SLP-76 to amino acid residues 224–244. Through the use of specific antisera directed against SLP-76, we demonstrate that this molecule becomes rapidly tyrosine phosphorylated after TCR stimulation and associates with several additional tyrosine phosphoproteins. Additionally, in vitro experiments demonstrate that the SH2 domain of SLP-76 associates with a serine/threonine kinase after TCR ligation. Finally, we demonstrate that

transient overexpression of SLP-76 results in dramatically enhanced TCR-mediated induction of the nuclear factor of activated T cells (NFAT) and IL-2 promoter activity. These results implicate SLP-76 as an important molecule in T cell activation.

Materials and Methods

Cell Culture and Antibodies. The human Jurkat T cell line was maintained in RPMI 1640 supplemented with 10% FCS, penicillin (1,000 U/ml), streptomycin (1,000 U/ml), and glutamine (20 mM). The following antibodies were used in this study: anti-TCR mAb C305 ([22]; gift of A. Weiss, University of California, San Francisco, CA); anti-TCR mAb OKT3 (American Type Culture Collection, Rockville, MD); antiphosphotyrosine mAb 4G10 (gift of B. Drucker, University of Oregon, Portland, OR); anti-Grb2 mAb (Upstate Biotechnology, Inc., Lake Placid, NY); and anti-flag mAb M2 (International Biotechnologies Inc., New Haven, CT). Anti–SLP-76 sheep antiserum 0083 was generated against amino acids 136–235 of human SLP-76 expressed as a GST fusion protein. For immunoprecipitation, the antiserum was conjugated to gamma bind plus Sepharose (Pharmacia, Uppsala, Sweden) and used at ~0.5 μl/20 × 10^6 cell equivalents. The antiserum was used at 1:500 for Western blotting.

cDNA Constructs and Fusion Proteins. SLP-76 cDNA was amplified by PCR using reverse-transcribed Jurkat mRNA as a template. This cDNA, which was subcloned into pBluescript (Stratagene Inc., La Jolla, CA) to generate the plasmid pBS/SLP-76/myc, possesses a COOH-terminal myc epitope tag (EQKLISEEDL) and a silent mutation introducing a HindIII restriction site at amino acids 229–230. SLP-76 cDNA with an NH$_2$-terminal flag epitope tag (DYKDDDDK) was generated by PCR using pBS/SLP-76/myc as a template and subcloned into the pEF-BOS mammalian expression vector ([23]; gift of D. Cantrell, Imperial Cancer Research Foundation, London, UK) to generate the plasmid pEF/flag/SLP-76 WT. This SLP-76 cDNA no longer possesses the myc COOH-terminal epitope tag. The flag/SLP-76 DNA encoding a deletion of amino acids 224–244 was generated by PCR using flag/SLP-76 DNA as a template and subcloned into pEF-BOS to generate the plasmid pEF/flag/SLP-76 Δ224-244. SLP-76 DNA possessing the R448K mutation was generated by overlap extension PCR using pBS/SLP-76/myc cDNA as a template. This cDNA possesses a silent mutation introducing a KpnI restriction site at amino acids 443–444. The resulting PCR product was subcloned into the plasmid pEF/flag/SLP-76 WT after removal of the corresponding wild-type sequences, resulting in the plasmid pEF/flag/SLP-76 R448K. The GST/SLP-76 fusion proteins consisting of the indicated amino acids were generated by PCR using reverse-transcribed Jurkat mRNA as a template and subcloned into either the pGEX-2TK or pGEX-3X bacterial expression vectors (Pharmacia). The GST/SLP-76 SH2 domain possessing the loss-of-function R448K mutation was generated by subcloning the overlap extension PCR product described above into the pGEX-3X expression vector. The GST/Grb2, GST/Grb2 SH2, and GST/Grb2 SH2 R86K constructs were generated by PCR and subcloned into pGEX-2TK. GST fusion proteins were induced and affinity purified as described (24).

Protein Precipitations and Immunoprecipitations. For each experiment, Jurkat cells were left unstimulated or were stimulated with anti-TCR mAb (C305, ascites 1:1,000) for 1 min unless otherwise noted. Cell lysates prepared in NP-40 lysis buffer (1% NP-40,

150 mM NaCl, 10 mM Tris, pH 7.4) including protease (50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin A, 1 mM PMSF) and phosphatase (400 nM sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate) inhibitors were then subjected to precipitation with the indicated GST fusion proteins or with the indicated antibodies for 2 h at 4°C. Resulting protein complexes were then washed extensively in high salt lysis buffer (500 mM NaCl), resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated mAb or antisera, followed by a horseradish peroxidase–conjugated 2° antibody (Bio-Rad Laboratories, Hercules, CA). Immunoreactive proteins were detected by ECL (Amersham Corp., Arlington Heights, IL).

In Vitro Kinase Assay and Phosphoamino Acid Analysis. Following the standard immunoprecipitation procedure, samples were washed further in 500 mM LiCl, 5 mM Tris, pH 7.4, and once in water. The samples were then resuspended in kinase reaction buffer (10 mM MnCl$_2$, 20 mM Tris, pH 7.4) plus [^{32}P]γATP (10 μCi/sample) for 10 min at room temperature, washed twice with water, subjected to SDS-8% PAGE, and visualized by autoradiography. The ~100-kD band was excised and subjected to phosphoamino acid analysis as described (25). Briefly, the sample was rehydrated in 50 mM NH$_4$HCO$_3$ and extracted twice with 0.1% SDS. The resulting supernatants were then precipitated with 20% TCA, acid hydrolyzed in 50 μl of 5.7 M HCl at 110°C for 10 min, and dried. The hydrolysate was then resuspended in running buffer (2.5% formic acid, 7.8% acetic acid, pH 1.9) with standards and subjected to TLC with a Hunter Thin Layer Electrophoresis System (CBS Scientific Co., Del Mar, CA). Standards were visualized with 0.25% ninhydrin, and the samples were detected by autoradiography.

Transient Transfections and Luciferase Assays. For transient transfections, log-phase Jurkat cells were electroporated with 40 μg of the indicated plasmid construct in serum-free RPMI at a density of 10^7 cells/400 μl per cuvette with a gene pulser (Bio-Rad Laboratories) set at 250 V and 960 μF. For transient transfections before luciferase assays, log-phase Jurkat cells were cotransfected with either 40 μg of pEF/flag/SLP-76 plasmid or empty vector DNA, plus 20 μg of the indicated reporter construct (either NFAT-luc, gift of G. Crabtree, Stanford University, Palo Alto, CA; or pIL-2luc2kb, gift of R. Abraham, Mayo Clinic, Rochester, MN), in serum-free RPMI at a density of 10^7 cells/400 μl per cuvette with the conditions specified above. After electroporation, the cells were transferred to complete RPMI and incubated at 37°C for 24 h. Triplicates of 5 × 10^5 viable cells were then stimulated as indicated for 8 h at 37°C and subsequently assayed for luciferase activity as described (26). Briefly, cells were lysed in 100 μl luciferase lysis buffer (1% Triton X-100, 110 mM K$_2$HPO$_4$, 15 mM KH$_2$PO$_4$, 5 mM dithiothreitol, pH 7.8) for 10 min at room temperature. The lysate was then added to 100 μl of 2× luciferase assay buffer (220 mM K$_2$HPO$_4$, 30 mM KH$_2$PO$_4$, 20 mM MgCl$_2$, 10 mM ATP, pH 7.8), and luciferase activity was quantitated with a luminometer (Monolight 2010; Analytical Luminescence Laboratory, San Diego, CA) immediately after the addition of 100 μl of 1 mM luciferin (Sigma Chemical Co., St. Louis, MO).

Results and Discussion

Grb2 Associates with SLP-76 from Resting Cells. Work from our laboratory and others has demonstrated that Grb2 asso-
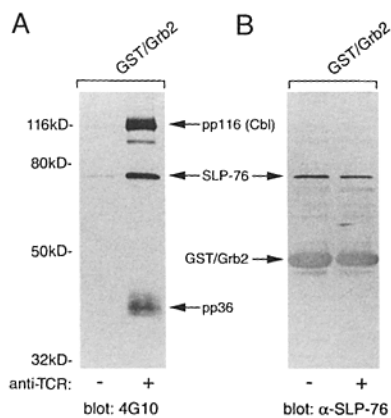
Figure 1. SLP-76 from resting and TCR-stimulated T cells associates with Grb2. NP-40 lysates of resting or TCR-stimulated Jurkat T cells were precipitated with a full-length GST/Grb2 fusion protein (10 μg fusion protein, 20 × 10⁶ cell equivalents/condition), and the resulting protein complexes were washed, resolved by SDS-10% PAGE, transferred to nitrocellulose, and immunoblotted with 4G10 antiphosphotyrosine mAb (*A*), or anti-SLP-76 antisera (*B*). The migrations of SLP-76, pp36, and pp116 (Cbl) are as indicated. The anti–SLP-76 antiserum was generated against a GST/SLP-76 fusion protein; therefore it also detects the GST/Grb2 fusion protein in *B*.

ciates with T lymphocyte tyrosine phosphoproteins of 36, 76, and 116 kD, both in vitro and in vivo (Fig. 1 *A* and references [17–20]). Recently we purified the 76-kD protein and isolated its cDNA, which encodes a novel hematopoietic cell-specific protein of 533 amino acids we have named

SLP-76 (21). Specific antisera generated against SLP-76 recognizes a 76-kD protein precipitated by GST/Grb2 from lysates of resting and TCR-stimulated Jurkat T cells (Fig. 1 *B*), demonstrating formally that the association between Grb2 and SLP-76 is independent of SLP-76 tyrosine phosphorylation.

Mapping of the Grb2-binding Site on SLP-76. Previous work has demonstrated that the association between Grb2 and SLP-76 is mediated via one or both of the Grb2 SH3 domains (17, 18). We therefore reasoned that the SLP-76 Grb2-binding site most likely is located within the proline-rich central domain of SLP-76. To map the Grb2-binding site within SLP-76, we subjected Jurkat cell lysates to precipitation with several GST/SLP-76 fusion proteins encompassing the central proline-rich domain of this molecule (Fig. 2 *A*). As shown, the fusion proteins that contain SLP-76 amino acid residues 225–267 precipitate Grb2 (Fig. 2 *A*, lanes *2–4*), suggesting that the Grb2-binding site is located within the middle third of the SLP-76 proline-rich domain. In confirmation of this finding, fusion proteins containing SLP-76 amino acids 225–265 also associate with Grb2 (Fig. 2 *B*, lanes *1* and *2*). Removal of ≥10 amino acids from the NH₂ terminus of this sequence completely abrogates Grb2 binding (lanes *3–5*), demonstrating that amino acids 225–235 of SLP-76 are required for its association with Grb2. However these amino acids are not sufficient for Grb2 binding because the fusion protein consisting of SLP-76 amino acids 136–235 does not associate with Grb2 (Fig. 2 *A*, lane *1*), suggesting that the amino acids im-
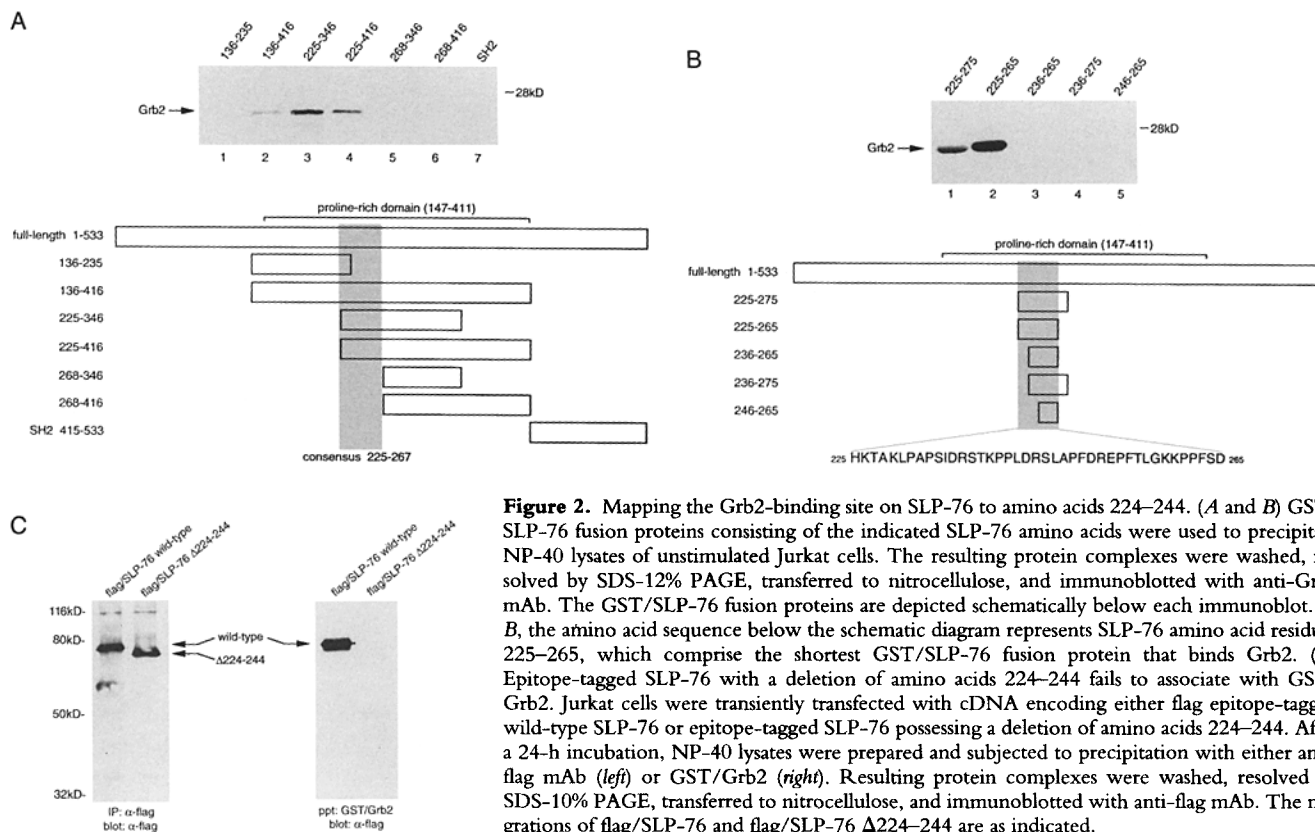

Figure 2. Mapping the Grb2-binding site on SLP-76 to amino acids 224–244. (*A* and *B*) GST/SLP-76 fusion proteins consisting of the indicated SLP-76 amino acids were used to precipitate NP-40 lysates of unstimulated Jurkat cells. The resulting protein complexes were washed, resolved by SDS-12% PAGE, transferred to nitrocellulose, and immunoblotted with anti-Grb2 mAb. The GST/SLP-76 fusion proteins are depicted schematically below each immunoblot. In *B*, the amino acid sequence below the schematic diagram represents SLP-76 amino acid residues 225–265, which comprise the shortest GST/SLP-76 fusion protein that binds Grb2. (*C*) Epitope-tagged SLP-76 with a deletion of amino acids 224–244 fails to associate with GST/Grb2. Jurkat cells were transiently transfected with cDNA encoding either flag epitope-tagged wild-type SLP-76 or epitope-tagged SLP-76 possessing a deletion of amino acids 224–244. After a 24-h incubation, NP-40 lysates were prepared and subjected to precipitation with either anti-flag mAb (*left*) or GST/Grb2 (*right*). Resulting protein complexes were washed, resolved by SDS-10% PAGE, transferred to nitrocellulose, and immunoblotted with anti-flag mAb. The migrations of flag/SLP-76 and flag/SLP-76 Δ224–244 are as indicated.
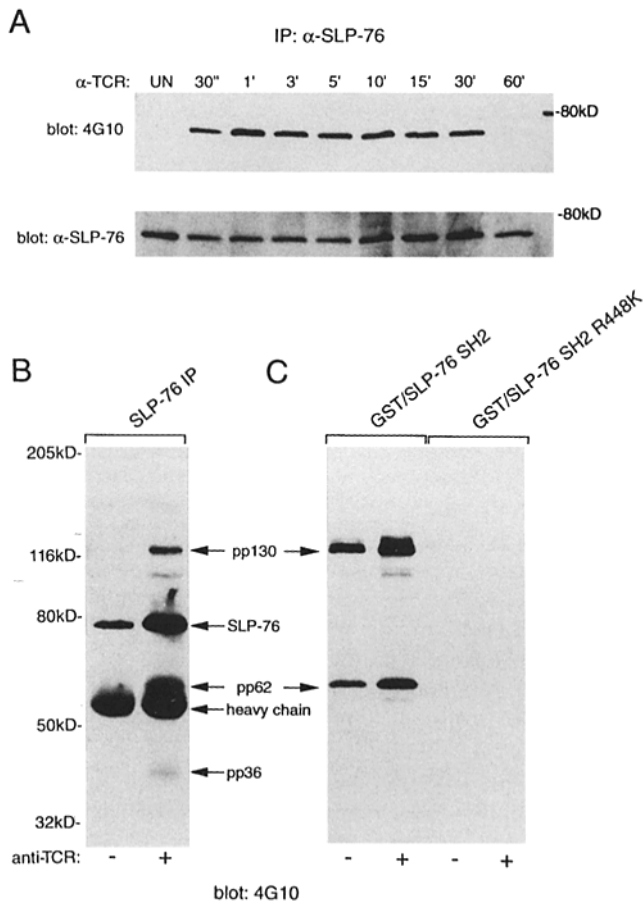
A

IP: α-SLP-76

α-TCR: UN 30" 1' 3' 5' 10' 15' 30' 60'

blot: 4G10   ─■-80kD

blot: α-SLP-76   ─-80kD

B   **C**

SLP-76 IP   GST/SLP-76 SH2   GST/SLP-76 SH2 R448K

205kD-

116kD-   ←── pp130 ──→

80kD-   ←── SLP-76

50kD-   ←── pp62 ──→
                 ←── heavy chain

                 ←── pp36

32kD-

anti-TCR: -   +   -   +   -   +

blot: 4G10

Figure 3. SLP-76 becomes tyrosine phosphorylated and associates with several additional proteins after TCR ligation. (*A*) Time course of TCR-induced SLP-76 tyrosine phosphorylation. Jurkat T cells were left unstimulated or were stimulated with anti-TCR mAb for the lengths of time indicated. NP-40 lysates were then prepared and subjected to immunoprecipitation with anti–SLP-76 antisera, and the resulting protein complexes were washed, resolved by SDS-8% PAGE, transferred to nitrocellulose, and immunoblotted with 4G10 antiphosphotyrosine mAb (*top*). Subsequently, the immunoblot was stripped and reprobed with anti–SLP-76 antisera (*bottom*). (*B* and *C*) SLP-76 associates with several tyrosine phosphoproteins after TCR ligation. NP-40 lysates of resting or TCR-stimulated Jurkat T cells were precipitated with either anti–SLP-76 antisera (75 × 10⁶ cell equivalents per condition) or the indicated GST/SLP-76 SH2 domain fusion proteins (10 μg fusion protein, 25 × 10⁶ cell equivalents/condition), and the resulting protein complexes were washed, resolved by SDS-8% PAGE, transferred to nitrocellulose, and immunoblotted with 4G10 antiphosphotyrosine mAb. The migrations of SLP-76, pp36, pp62, and pp130 are as indicated.

mediately following residue 235 also contribute to the Grb2 binding site. In support of this notion, a synthetic peptide composed of SLP-76 amino acid residues 229–246 blocks the association between Grb2 and SLP-76 in vitro (not shown). To verify that our experiments with GST/SLP-76 fusion proteins correctly localized the Grb2-binding site, we constructed and transiently expressed in Jurkat cells cDNAs encoding epitope-tagged wild-type SLP-76 and an epitope-tagged SLP-76 mutant possessing a deletion of amino acids 224–244 (Fig. 2 C). As shown, although both of these proteins are expressed similarly, only the wild-type molecule is precipitated by GST/Grb2, demonstrating
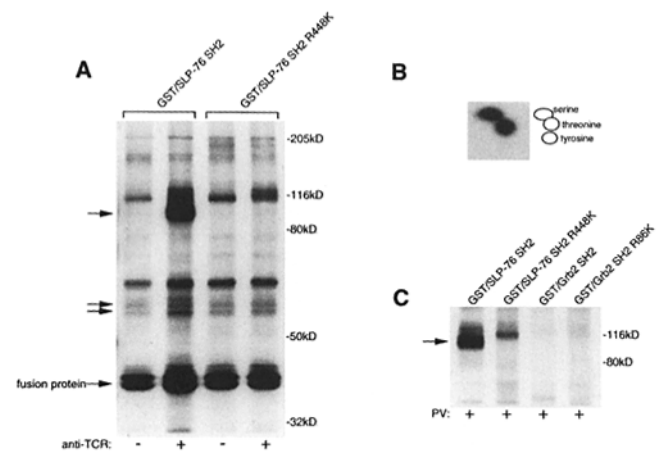
A   **B**

GST/SLP-76 SH2   GST/SLP-76 SH2 R448K

-205kD

-116kD

-80kD

-50kD

fusion protein→

-32kD

anti-TCR: -   +   -   +

C

GST/SLP-76 SH2   GST/SLP-76 SH2 R448K   GST/Grb2 SH2   GST/Grb2 SH2 R86K

-116kD

-80kD

PV: +   +   +   +

Figure 4. (*A*) In vitro kinase assay of GST/SLP-76 SH2 domain fusion protein precipitations. NP-40 lysates of resting or TCR-stimulated (OKT3, ascites 1:1,000, 2 min) Jurkat T cells were precipitated with the indicated GST/SLP-76 SH2 domain fusion protein (10 μg fusion protein, 75 × 10⁶ cell equivalents/condition), and the resulting protein complexes were washed and subjected to an in vitro kinase assay with ³²P-γATP. The resulting radiolabeled reaction products were then resolved by SDS-8% PAGE and detected by autoradiography. (*B*) Phosphoamino acid analysis of the 100-kD phosphoprotein from *A*. The migrations of the phosphoserine, phosphothreonine, and phosphotyrosine standards are as shown. (*C*) The serine/threonine kinase activity that associates with the SH2 domain of SLP-76 is not precipitated by the SH2 domain of Grb2. NP-40 lysates of pervanadate-stimulated Jurkat T cells were precipitated with the indicated GST/SH2 domain fusion protein (10 μg fusion protein, 50 × 10⁶ cell equivalents/condition), and the resulting protein complexes were washed and subjected to an in vitro kinase assay with ³²P-γATP. The resulting radiolabeled reaction products were then resolved by SDS-9% PAGE and detected by autoradiography.

that the Grb2-binding site on SLP-76 is located within amino acids 224–244.

Tyrosine Phosphorylation Time Course and Association of SLP-76 with Several Phosphoproteins after TCR Stimulation. SLP-76 was identified originally as a Grb2-associated tyrosine phosphoprotein. To demonstrate formally that SLP-76 is a substrate of the TCR-activated PTKs, we subjected anti–SLP-76 immunoprecipitates from lysates of resting and TCR-stimulated Jurkat cells to antiphosphotyrosine Western blotting (Fig. 3 *A*). As shown, SLP-76 undergoes an increase in tyrosine phosphorylation within 30 s of TCR ligation, which peaks by 1 min, and returns nearly to baseline by 60 min of stimulation. Additionally, when increasing numbers of Jurkat cells are used, tyrosine phosphoproteins of 36, 62, and 130 kD coimmunoprecipitate with SLP-76 (Fig. 3 *B*). The 36-kD protein likely associates with SLP-76 indirectly, via its association with Grb2. pp62 and pp130 interact with the SH2 domain of SLP-76 as demonstrated by their association with an SLP-76 SH2 domain GST fusion protein (Fig. 3 *C*). Mutation of the predicted phosphate-binding arginine residue of the SLP-76 SH2 domain to lysine (R448K) completely abrogates binding of pp62 and pp130, suggesting that the association between these molecules is dependent on tyrosine phosphorylation of pp62 and pp130 (Fig. 3 *C*). Western blotting of GST/SLP-76 SH2 domain precipitations with antibodies against known
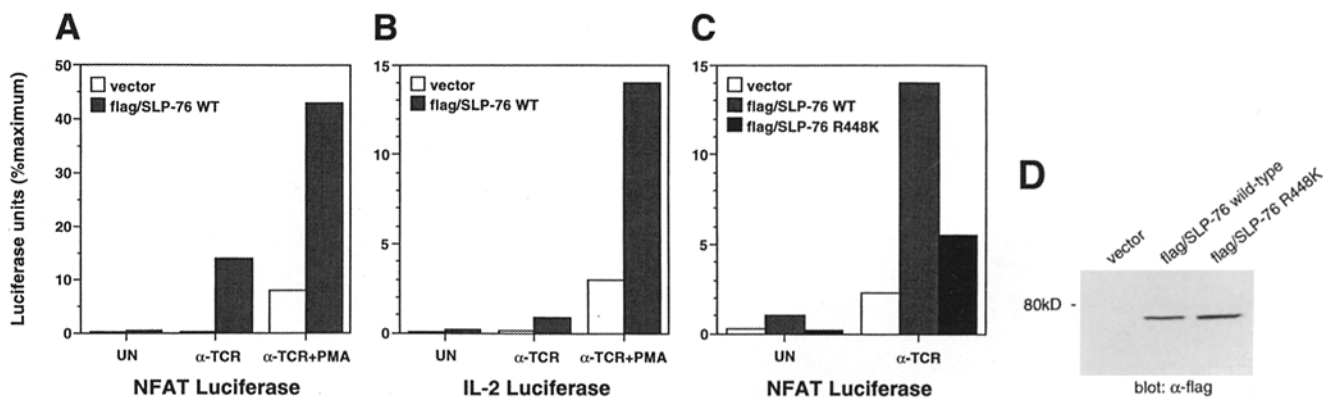
Figure 5. Overexpression of SLP-76 greatly augments TCR-mediated induction of NFAT and IL-2 promoter activity. (*A*) Jurkat cells were transiently cotransfected with an NFAT–luciferase reporter construct together with either empty vector or epitope-tagged SLP-76 cDNA. The cells were then stimulated with media, anti-TCR mAb C305 (ascites 1:1,000), or C305 plus PMA (50 ng/ml) and subsequently assayed for luciferase activity. The results are expressed as the percentage of arbitrary luciferase units (mean of triplicates) induced by the stimulation condition compared with the maximal stimulation for each transfection condition induced by PMA (50 ng/ml) plus ionomycin (1 μM) treatment. Maximal stimulations for this experiment were ~3 × 10^6 light units for both transfection conditions. (*B*) Jurkat cells were transfected and analyzed as in *A* except the reporter construct used was IL-2 luciferase. Maximal stimulations were 2 × 10^6 light units for the empty vector transfection and 1.4 × 10^6 light units for the SLP-76 transfection. (*C*) A functional SH2 domain of SLP-76 is required for its ability to maximally augment TCR-mediated induction of NFAT activity. Jurkat cells were transiently cotransfected with an NFAT–luciferase reporter construct together with either empty vector, epitope-tagged wild-type SLP-76 cDNA, or epitope-tagged SLP-76 cDNA possessing the R448K mutation. The cells were then stimulated with either media or anti-TCR mAb C305 (ascites 1:1,000) and analyzed as in *A*. Maximal stimulations were 0.9 × 10^6 light units for the empty vector transfection and 1.2 × 10^6 light units for the SLP-76 transfections. In this experiment, 10 μg more R448K than wild-type cDNA was transfected to ensure greater-than or equal-to expression of mutant protein compared with wild-type. (*D*) Expression levels of the SLP-76 constructs from *C*. NP-40 lysates (2 × 10^6 cell equivalents/condition) from each transfection condition were resolved by SDS-8% PAGE, transferred to nitrocellulose, and immunoblotted with anti-flag mAb. The migrations of the SLP-76 constructs are as indicated.

proteins of similar molecular mass demonstrates that pp62 is not p62/SAM, and that pp130 is not Cbl, GAP, focal adhesion kinase (FAK), or p130^CAS (not shown). We are currently pursuing the identification of these molecules.

To characterize further the properties of proteins that associate with the SLP-76 SH2 domain, we subjected GST/ SLP-76 SH2 domain fusion protein precipitations from resting and TCR-stimulated Jurkat cell lysates to an in vitro kinase assay with [32P]γATP (Fig. 4 *A*). As shown, TCR stimulation results in increased protein kinase activity detected in the wild-type fusion protein precipitation evidenced by the increased phosphorylation of several protein species as indicated. Interestingly, when the phosphoprotein migrating at ~100 kD was excised and subjected to phosphoamino acid analysis, it was found to contain phosphoserine and phosphothreonine, but not phosphotyrosine (Fig. 4 *B*). This kinase activity appears to be specific for the SH2 domain of SLP-76, as the 100-kD phosphoprotein was not detected when a similar experiment was performed using GST/Grb2 SH2 domain fusion protein precipitations (Fig. 4 *C*). As expected, the wild-type GST/Grb2 SH2 domain fusion protein associated with the previously described 36-kD tyrosine phosphoprotein as demonstrated by antiphosphotyrosine Western blotting (not shown). These results suggest that a serine/threonine kinase associates either directly or indirectly with the SH2 domain of SLP-76 after TCR stimulation.

Overexpression of SLP-76 Dramatically Enhances TCR-mediated NFAT and IL-2 Promoter Activity. Our data demonstrating that SLP-76 becomes tyrosine phosphorylated after TCR ligation, coupled with the observation that SLP-

76 associates with several other potential signaling proteins, suggest that this molecule plays a role in TCR-mediated signal transduction. One important consequence of TCR ligation is the activation of the IL-2 gene, which is mediated in part by binding of transcription factors, such as NFAT, to the IL-2 promoter. To determine whether SLP-76 plays a role in this process, we investigated the effect of transient SLP-76 overexpression on TCR-mediated induction of NFAT activity through the use of an NFAT-driven luciferase reporter system (Fig. 5 *A*). As shown, overexpression of SLP-76 greatly augments NFAT activity induced by both TCR ligation and TCR ligation plus phorbol ester stimulation. Western blotting of cell lysates demonstrated that transient transfection of SLP-76 results in its overexpression by severalfold (not shown). To determine if SLP-76 overexpression augments transcription from the entire IL-2 promoter, we performed a similar assay using instead a luciferase reporter construct driven by 2 kb of IL-2 promoter sequence (Fig. 5 *B*). Similar to what was observed with the NFAT promoter, SLP-76 overexpression enhances IL-2 promoter activity induced by both TCR and TCR plus phorbol ester stimulation. These data suggest that SLP-76 plays a key role in linking the TCR with an important distal event in T cell activation.

We next investigated whether the associations between SLP-76 and molecules that bind its SH2 domain are required for its function in TCR-mediated signaling. As demonstrated above, the R448K mutation abrogates binding of phosphotyrosine-containing proteins to the SLP-76 SH2 domain; therefore we investigated the effect of this loss-of-binding mutation on the ability of SLP-76 to aug-

ment TCR-mediated NFAT activity. As shown, overexpression of SLP-76 possessing the R448K mutation is much less effective at enhancing NFAT activity induced by TCR ligation when compared with wild-type SLP-76 (Fig. 5 C). That this effect is not due to lower level expression of the mutant protein was confirmed by antiepitope Western blotting of cell lysates (Fig 5 D). These data demonstrate that a functional SH2 domain contributes to the ability of SLP-76 to participate in T cell activation.

SLP-76 was identified originally as a phosphoprotein that associates with Grb2 both in vitro and in vivo (17, 18, 21). In this report, we map the region of SLP-76 responsible for its association with Grb2 and demonstrate that this interaction is not dependent upon tyrosine phosphorylation. We show further that after TCR stimulation, SLP-76 becomes rapidly tyrosine phosphorylated and associates in vivo with several unidentified tyrosine phosphoproteins. Two of these proteins, pp62 and pp130, apparently bind the SH2 domain of SLP-76, as evidenced by their association with a GST/SLP-76 SH2 domain fusion protein. Interestingly, the SLP-76 SH2 domain also appears to bind a serine/threonine kinase after TCR ligation. These findings suggested that SLP-76 plays an important role in linking the TCR with distal events in T cell activation. We tested this hypothesis by as-

sessing the effect of SLP-76 overexpression on TCR-mediated induction of NFAT and IL-2 promoter activity and found that these important distal events of T cell activation are indeed dramatically enhanced by SLP-76 overexpression. We demonstrate further that this function of SLP-76 is at least partially dependent on its associations with molecules that bind its SH2 domain, as overexpression of SLP-76 with a nonfunctional SH2 domain is less effective at enhancing TCR-mediated induction of NFAT activity than is wild-type SLP-76. As these events in wild-type T cells require the coordination of several signaling pathways, including those mediated by PLCγ1 and Ras (27–29), it is not yet clear in which biochemical pathway SLP-76 is functioning. Likewise, it is not yet clear which features of SLP-76 are necessary for its function in these processes. Now that we have identified regions of SLP-76 responsible for its associations with other potential signaling molecules, it will be possible to perform a detailed structure/function analysis of this molecule. Additionally, it will be important to identify tyrosine phosphorylation sites of SLP-76 and determine their contribution to its function. These experiments should help to elucidate further the role of SLP-76 in the process of T cell activation.

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