SLP-76 and Vav Function in Separate, but Overlapping Pathways to Augment Interleukin-2 Promoter Activity

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SLP-76 and Vav, two hematopoietic cell specific molecules, are critical for T cell development and activation. Following T cell antigen receptor stimulation, SLP-76 and Vav both undergo tyrosine phosphorylation and associate with each other via the SH2 domain of Vav and phosphorylated tyrosines of SLP-76. Furthermore, SLP-76 and Vav have a synergistic effect on interleukin (IL)-2 promoter activity in T cells. In this report, we show that two tyrosines, Tyr-113 and Tyr-128, of SLP-76 are required for its binding to Vav, both in vitro and in intact cells. Surprisingly, we find also that the interaction between SLP-76 and Vav is not required for their cooperation in augmenting IL-2 promoter activity, as the two molecules appear to function in different signaling pathways upstream of IL-2 gene expression. Overexpression of SLP-76 in the Jurkat T cell line potentiates the activities of both nuclear factor of activated T cells and AP-1 transcription factors. In contrast, overexpression of Vav leads to enhanced nuclear factor of activated T cells activity without affecting AP-1. Additionally, overexpression of Vav, but not SLP-76, augments CD28-induced IL-2 promoter activity. These findings suggest that the synergy between SLP-76 and Vav in regulating IL-2 gene expression reflects the cooperation between different signaling pathways.

Recognition of specific antigen by the T cell antigen receptor (TCR)1 initiates biochemical changes including protein tyrosine phosphorylation, calcium influx, metabolism of phospholipids, and activation of the Ras/MAPK cascade (1, 2). Together with signaling events initiated by engagement of co-receptors on the T cell, these biological second messengers lead to cytoskeletal rearrangement, proliferation, and differentiation, or conversely programmed cell death (3–5). Activation of protein tyrosine kinases (PTKs) of the Src and Syk families is the most membrane-proximal biochemical event known following TCR engagement (6). Phosphorylation of cellular proteins by these kinases is essential for all subsequent TCR-initiated signaling pathways. PTK activation may result in downstream signals through direct phosphorylation and modulation of the catalytic activities of effector molecules (such as phospholipase C-γ1) or the formation of signaling complexes by the interaction of proteins newly phosphorylated on tyrosine residues with other Src homology 2 (SH2) or phosphotyrosine-binding domain-containing proteins within the cell. A widely studied example of this in T cells is the recruitment of ZAP-70 to the newly phosphorylated tyrosines of immune receptor activation motifs (ITAMs) on the TCR ζ and CD3 chains (7, 8).

Given the central importance of PTKs in T cell activation, a number of laboratories have attempted to identify substrates of these PTKs in an effort to gain insight into how biochemical signals are integrated. Among the substrates identified are the hematopoietic cell-specific molecules SH2 domain containing leukocyte phosphoprotein of 76 kDa (SLP-76) and Vav, both of which play critical roles in the regulation of TCR signals (9, 10). SLP-76 is an adapter protein which is comprised of three motifs allowing for protein-protein interactions: an amino-terminal acidic region containing tyrosine phosphorylation sites (11), a middle proline-rich motif that binds to the SH3 domain of Grb2 family members (9, 12), and a carboxyl-terminal SH2 domain that associates with SLP-76-associated phosphoprotein of 130 kDa (SLAP-130 or FYB, for Fyn-binding protein) and another unidentified 62-kDa phosphoprotein (13–15). The importance of SLP-76 in T cell signaling has been demonstrated in experiments in the Jurkat human T cell leukemia line and in mice made deficient in SLP-76 expression through homologous recombination. When SLP-76 is overexpressed in Jurkat cells, there is a dramatic augmentation of TCR-mediated activation of the full-length IL-2 promoter or a reporter construct driven by the nuclear factor of activated T cells (NFAT) element from the IL-2 promoter (9, 16). Each of the three structural domains of SLP-76 are essential for this enhanced promoter activity (17). Furthermore, TCR-mediated signals are abrogated in a mutant variant of Jurkat which has lost expression of SLP-76 (18). Studies of SLP-76-deficient mice indicate also that this adapter protein is critical for signaling via the pre-TCR, as these mice exhibit arrest of thymocyte development at the pro-T3, CD3+ CD4+ CD8+ stage (19, 20).

Vav is a guanine nucleotide exchange factor which acts on members of the Ras/Rho family of small GTP-binding proteins (21, 22). Vav has a pleckstrin homology, a Dbl homology, an SH2, and two Src homology 3 (SH3) domains (23, 24). The Dbl homology domain of Vav is responsible for its nucleotide exchange activity while the pleckstrin homology domain regulates interactions with inositol phospholipids (25). The SH2 and SH3 domains mediate the interaction of Vav with other signaling molecules, including SLP-76 (26–29). Like SLP-76, Vav is critical for effective TCR signaling. Its overexpression in Jurkat T cells also augments the transcriptional activity of the IL-2 promoter following TCR ligation (10). T cells from Vav-deficient mice do not respond effectively to TCR stimulation.
SLP-76 and Vav Function in Separate Signaling Pathways

SLP-76 and Vav function in separate signaling pathways. The interaction between SLP-76 and Vav depends on the SH2 domain of Vav and tyrosine phosphorylation of SLP-76. Both Tyr-113 and Tyr-128 are required for the interaction between SLP-76 and Vav. The interaction between SLP-76 and Vav is modulated by the SH2 domain of Vav and tyrosine phosphorylation.

RESULTS

Both Tyrosines 113 and 128 of SLP-76 Are Required for the SLP-76/Vav Interaction—The interaction between SLP-76 and Vav depends on the SH2 domain of Vav and tyrosine phosphorylation of SLP-76. Both Tyr-113 and Tyr-128 are required for the interaction between SLP-76 and Vav.

Experimental Procedures

Cell Culture and Antibodies—The human Jurkat T cell line was cultured in supplemented RPMI 1640 medium containing 10% fetal calf serum, penicillin (1,000 units/ml), streptomycin (1,000 units/ml), and L-glutamine (20 mM). For stimulation through the TCR, ascites of the clonotypic mAb C305 (1:1,000) was used for stimulating cells. Monoclonal antibody M2 recognizes the FLAG epitope (International Biotechnologies, New Haven, CT). Monoclonal antibody 9E10 recognizes the Myc epitope. Phosphotyrosine-containing proteins were detected by the 4G10 mAb (Upstate Biotechnology, Inc., Lake Placid, NY).

cDNA Constructs and Fusion Proteins—Wild-type SLP-76 and each of the mutants were cloned into modified pEF-BOS vector containing a sequence encoding the FLAG epitope on the amino terminus of the cDNAs, as described previously (17, 18). cDNAs encoding Vav and ERK2 with a Myc epitope on the amino terminus were also cloned into pEF-BOS vector (gifts of Dr. M. Weiss, University of California, San Francisco, CA). The NFAT luciferase reporter construct (NFAT-luc) was provided by Dr. G. Crabtree (Stanford University, Palo Alto, CA). The full-length IL-2 promoter-driven luciferase construct (pIL-2Luc2kb) was a gift of D. R. Abraham (Mayo Clinic, Rochester, MN). The AP-1 β-Gal construct (pAP-1-lacZ) was a gift of Dr. D. Mueller (University of Minnesota, Minneapolis, MN). The CD28RE luciferase reporter construct was provided by Dr. K. L. McGuire (San Diego State University, San Diego, CA). The NFAT luciferase reporter construct (NFAT-luc) was provided by Dr. S. Katsav (Lady Davis Institute, Jewish General Hospital, Montreal, Quebec, Canada). The GST fusion protein was induced and affinity purified as described (35).

Transfections—For transient transfection, 10^7 cells were electroporated at 250 V, 960 microfarads using a Gene Pulser (Bio-Rad) as described (33). In contrast, wild-type SLP-76 and the Y145F mutant still bind gates the binding of SLP-76 to the SH2 domain of Vav.

Immunoprecipitations, Protein Precipitations, and Immunoblots—Protein precipitations and immunoblots were performed as described previously (17). Briefly, Jurkat cells were transfected with pEF, pEF-SLP76, pEF/Vav plus pEF/myc/ERK2. 24 h later, transfected cells were left unstimulated or stimulated with anti-TCR mAb C305 or phorbol myristate acetate (PMA), lysed in Triton X-100 lysis buffer, and precipitated with anti-Myc antibody for 2 h at 4 °C after stimulation. The anti-Myc immune complexes were washed extensively and suspended in kinase buffer (20 mM Tris, pH 7.6, 13 mM MgCl2, and 1.5 mM EGTA) containing myelin basic protein (MBP, 1 mg/ml) and [γ-32P]ATP (0.22 μCi/sample), incubated at room temperature for 15 min, and subjected to SDS-PAGE. Each gel was stained with Coomassie Blue to assure equal loading of the MBP substrate and phosphorylation of MBP was visualized by autoradiography and quantitated by densitometry using the NIH Image computer program.

In the experiments described in this report we investigated these questions first by identifying the tyrosines of SLP-76 which are responsible for the interaction with Vav both in vitro and in intact Jurkat cells. Using this information, we performed a series of co-transfection assays and determined that the intermolecular interaction between SLP-76 and Vav is required for their functional synergy. This finding suggested the hypothesis that overexpression of SLP-76 versus Vav may impact distinct signaling pathways in T cells. Experiments are presented demonstrating that these two molecules modulate distinct signaling cascades to link T cell surface receptors with IL-2 gene expression.
proper subcellular location where Vav executes its role as a guanine nucleotide exchange factor. If this were true, the synergy between SLP-76 and Vav should require their interaction.

To test this possibility, we transfected cDNA encoding SLP-76, but not Vav, Augments TCR-induced AP-1 Activity—Our observation that the association between SLP-76 and Vav is not required for their functional cooperation suggests the possibility that these two proteins modulate different TCR-induced signaling cascades. We and others have found that when overexpressed individually, both SLP-76 and Vav augment activity of the NFAT region of the IL-2 promoter (9, 10). Work from several laboratories has documented by Western analysis. SLP-76 expression level is not changed with co-overexpression of Vav (data not shown). These data are representative of three independent experiments.

We have shown that in the Jurkat T cell line, SLP-76 overexpression augments TCR-induced activation of a β-galactosidase reporter driven by 5 copies of an AP-1 response element (17). To test whether overexpression of Vav has a similar impact on AP-1 function, we transfected Jurkat cells with the AP-1 reporter construct along with either SLP-76 or Vav and measured β-galactosidase activity in resting and stimulated cells. As shown in Fig. 3, TCR or PMA stimulation results in only a modest increase in AP-1 reporter activity in cells transfected with vector control. TCR stimulation of cells transfected with SLP-76 cDNA results in marked augmentation of reporter activity. As expected, SLP-76 overexpression does not impact on PMA-stimulated AP-1 function as SLP-76 is not tyrosine phosphorylated following PMA stimulation (Fig. 3 and data not shown). In contrast to results seen with SLP-76, overexpression of Vav does not increase TCR stimulation AP-1 activity above that seen with the vector transfected control cells. Thus it appears that although both SLP-76 and Vav can modulate some TCR signals, those leading to AP-1 activation are affected by SLP-76 but not Vav.

SLP-76, but Not Vav, Leads to ERK Activation—There is considerable evidence to suggest that extracellular signal-regulated kinase (ERK) family members stimulated by TCR ligation function upstream of AP-1 activation (40, 42, 43). We have found previously that overexpression of SLP-76 augments TCR induced activity of ERK2 (17). To investigate if Vav has a

**FIG. 2.** The synergy between SLP-76 and Vav does not require their interaction. A luciferase reporter construct driven by three copies of the NFAT element of IL-2 promoter was transfected into Jurkat cells alone with control vector or cDNA encoding SLP-76 (WT, Y113F, Y128F, and Y145F), Vav, or SLP-76 plus Vav. 24 h after transfection, cells were left untreated (UN; □) or stimulated with anti-TCR mAb C305 (TCR; ■) or PMA plus ionomycin at 37 °C for 8 h. Lysates were prepared and analyzed for luciferase activity. Results are shown as the percentage of the PMA plus ionomycin response (maximum) for each group. Expression of SLP-76 constructs and Vav constructs were documented by Western analysis. SLP-76 expression level is not changed with co-overexpression of Vav (data not shown). These data are representative of three independent experiments.
ERK2, but not SLP-76, impacts PMA-stimulated AP-1 activity with either SLP-76 or Vav. As predicted, overexpression of ERK2 alone or Vav does not appear to modulate TCR-stimulated ERK2, we reasoned that SLP-76 affects AP-1 indirectly via augmentation of AP-1 activity, overexpression of SLP-76 and ERK together should lead to additive enhancement of AP-1 activity. The β-Gal counts were normalized to the Rous sarcoma virus-luciferase controls to correct for transfection efficiency. Expression of transfected SLP-76 or Vav was documented by Western analysis with anti-FLAG (for SLP-76) or anti-Myc (for Vav) antibody (data not shown). This experiment is representative of five independent experiments.

We reasoned that if SLP-76 affects AP-1 indirectly via augmentation of ERK activity, overexpression of SLP-76 and ERK together should lead to additive enhancement of AP-1 activity in TCR-, but not PMA-stimulated cells. Furthermore, since Vav does not appear to modulate TCR-stimulated ERK2, we reasoned that overexpression of Vav plus ERK2 would not result in AP-1 function above that seen with overexpression of ERK2 alone. We tested these hypotheses in the experiments shown in Fig. 4. For this study, the AP-1 reporter construct was transfected into Jurkat cells along with cDNAs encoding SLP-76 or Vav. Cells were left untreated or stimulated via the TCR or PMA. Cellular lysates were then subjected to immunoprecipitation with anti-Myc and in vitro kinase assays were performed on each immune complex using MBP as an artificial substrate. Phosphorylation of MBP was quantitated by densitometry and normalized to PMA-induced MBP phosphorylation. Whereas overexpression of SLP-76 consistently enhances TCR-induced kinase activity of ERK2 to a modest degree, overexpression of Vav does not affect ERK2 enzymatic activity (data not shown).

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binding to tyrosine-phosphorylated residues within the SLP-76 amino terminus (28, 29, 37). In experiments presented in this study we found that although the binding is mediated by a single Vav SH2 domain, two SLP-76 tyrosines, Tyr-113 and Tyr-128, both appear essential for this association. This is particularly surprising since both Tyr-113 and Tyr-128 are found in identical sequence motifs (DYESP), either of which would be predicted to bind the Vav SH2. Although we are not yet sure why both SLP-76 tyrosines are required for Vav binding, several models come to mind. One possibility is that tyrosine phosphorylation of SLP-76 is sequential, and that phosphorylation of either tyrosine 113 or 128 is required for induced or maintained phosphorylation of the second residue. Thus, although only one tyrosine may be involved in direct binding to Vav, the second tyrosine is required for the phosphorylation of the Vav-binding tyrosine. However, while mutation of both tyrosines 113 and 128 abolishes TCR-induced tyrosine phosphorylation of SLP-76, mutants with either Tyr-113 or Tyr-128 mutated individually are still phosphorylated following TCR or pervanadate stimulation, making this explanation unlikely. A second possibility is that one phosphorylated tyrosine is responsible for directing the interaction with the Vav SH2 domain, while the second tyrosine is required for stabilizing this interaction, perhaps through maintaining a structural microenvironment or via an interaction with a third molecule. In this regard, SLP-76 was recently shown to simultaneously bind to the SH2 domains of Vav and the adapter protein, Nck, to form a trimolecular complex (55). Thus, it is possible that the SLP-76/Nck interaction is important for stabilizing the association between SLP-76 and Vav. Crystal or solution nuclear magnetic resonance determination of the structures of SLP-76, Vav, and Nck, when bound together, will be necessary to test this hypothesis.

On the surface, a second finding from our studies, that the synergy between SLP-76 and Vav does not appear to require their association, seems surprising. One potential explanation could be that the SLP-76 tyrosine mutants dimerize with endogenous, wild-type SLP-76 and thus interact with Vav indirectly. However, multiple experimental approaches in our laboratory have failed to demonstrate evidence for a SLP-76/Vav interaction, making this explanation unlikely. Moreover, our result is consistent with several other observations. First, when SLP-76 mutants Y113F and Y128F are overexpressed at high levels, they augment IL-2-NFAT promoter activity to a level similar to that of wild-type SLP-76 (11), suggesting that in this assay, SLP-76 can function independently of its ability to bind Vav. Second, SLP-76 and Vav synergize in augmenting basal IL-2-NFAT promoter activity in Jurkat cells, although under these conditions, SLP-76 is not phosphorylated and does not bind Vav (29). Third, it was reported that in the T cell hybridoma DC27.10, where there is no detectable association between SLP-76 and Vav (even following TCR stimulation), TCR ligation still induces IL-2 production (37). Finally, we have found recently that mice made deficient in SLP-76 expression by homologous recombination have a much more profound defect in T cell development than do mice which lack expression of Vav, suggesting that SLP-76 and Vav do not function entirely in the same pathway (19, 20, 30, 31, 56). Collectively, these observations, together with the experiments presented in our current study, suggest that the Vav/SLP-76 interaction is not essential for their functional synergy, at least for inducing IL-2 gene transcription. This conclusion led us to ask if SLP-76 and Vav may work together by modulating different signaling pathways in T cells.

There are several examples of how different pathways may enhance signals leading to IL-2 gene expression in T cells. One involves the cooperation between signals initiated from the TCR and those stimulated by engagement of CD28 (47). Another is the interdependence of signals leading to translocation.
Further insight into the potential mechanisms by which SLP-76 exerts its effects on TCR-mediated signaling have come from studies of the SLP-76 defective Jurkat mutant. In this cell line, TCR engagement results in activation of PTKs, but fails to couple effectively with the phosphatidylinositol as well as Ras signaling pathways (18). Thus, it appears that SLP-76, via its adaptor function, is critical for allowing TCR-stimulated PTK activation to result in optimal phosphorylation of phospholipase Cγ1 in addition to signals leading to ERK activation. It remains to be determined, however, exactly which protein-protein interactions mediated by SLP-76 are critical for these more downstream signals to occur.

Although the experiments in this study indicate that for TCR-stimulated IL-2 gene regulation, the interaction between SLP-76 and Vav does not appear to play a critical role, it is possible, and perhaps likely, that other T cell activation events are influenced by SLP-76 and Vav and require their interaction. For example, it has been reported that the interaction between SLP-76 and Vav may be required for TCR-induced PKC activation as well as the capping process (55). Since several studies have shown that Vav plays a critical role in formation of the cap following TCR stimulation (56, 58, 59), it will be important to study capping function in SLP-76-deficient T cells. We are currently working to reconstitute our SLP-76-deficient mice with wild-type and mutant variants of SLP-76 to begin to address this and other related questions.

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