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Role of Two Adaptor Molecules SLP-76 and LAT in the PI3K Signaling Pathway in Activated T Cells

Eun Kyung Shim,*1 Seung Hee Jung,*1 and Jong Ran Lee*1,†,‡

Previously, we identified p85, a subunit of PI3K, as one of the molecules that interacts with the N-terminal region of Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76). We also demonstrated that tyrosine phosphorylation either at the 113 and/or 128 position is sufficient for the association of SLP-76 with the Src homology 2 domain near the N terminus of p85. The present study further examines the role of the association of these two molecules on the activation of PI3K signaling cascade. Experiments were done to determine the role of SLP-76, either wild-type, tyrosine mutants, or membrane-targeted forms of various SLP-76 constructs, on the membrane localization and phosphorylation of Akt, which is an event downstream of PI3K activation. Reconstruction studies with these various SLP-76 constructs in a Jurkat variant cell line that lacks SLP-76 or linker for activation of T cells (LAT) show that the activation of PI3K pathway following TCR ligation requires both SLP-76 and LAT adaptor proteins. The results suggest that SLP-76 associates with p85 after T cell activation and that LAT recruits this complex to the membrane, leading to Akt activation. The Journal of Immunology, 2011, 186: 2926–2935.

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rc homology (SH) 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) is an adaptor protein that is comprised of three motifs allowing for protein–protein interactions: an N-terminal acidic region containing tyrosine phosphorylation sites, a middle proline-rich motif that binds to the SH3 domain of Grb2 family members, and a C-terminal SH2 domain (1). The importance of SLP-76 in T cell development and function has been well characterized in the experiments in the Jurkat human T cell leukemia line and in mice made deficient for SLP-76 expression by homologous recombination (2–9). During T cell activation, SLP-76 is recruited to the glycolipid-enriched membrane microdomains (GEMs), also called lipid rafts, upon which signaling complexes are formed (10, 11). After the ligation of TCR, a transmembrane adaptor protein in GEMs, linker for activation of T cells (LAT), is tyrosine-phosphorylated and recruits multiple signaling intermediates through their SH2 domains, including Grb2 (and associated Sos), phospholipase Cγ1, and p85, a subunit of PI3K (12–14). The recruitment of SLP-76 to GEMs through its indirect association with LAT by Grb2-related adaptor downstream of Src (Gads) binding is a critical event in the early TCR signaling (10, 11).

Previously, we identified p85 as one of the molecules that interacts with an N-terminal acidic region of SLP-76 (15). We also demonstrated that tyrosine phosphorylation either at the 113 or 128 position is sufficient for the association of SLP-76 with the SH2 domain near the N terminus of p85 (15). A regulatory subunit of PI3K, p85, contains two SH2 domains that are separated by an interacting domain, which binds with a catalytic subunit (16). Together with other protein-interaction domains in the catalytic and regulatory subunits, the SH2 domains recruit PI3K to membrane-associated signaling complexes after the activation of protein tyrosine kinases followed by TCR ligation (16). PI3K constitutes an active form of the enzyme by membrane targeting of the p110 catalytic subunit and generates phospholipid second messengers, such as phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 3,4,5-trisphosphate (PIP3) (16). Concurrently, the enzymes phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B/Akt are translocated to the plasma membrane by PIP3 binding, and PDK1 directs phosphorylation and activation of Akt ensues (16). Once phosphorylated on Thr308 and Ser473, fully activated Akt then dissociates from the plasma membrane and phosphorylates a variety of substrates, including glycogen synthase kinase 3β (Gsk3β) and FKHR (FOXO1) (17). Activation of these and other downstream molecules by Akt collectively results in signals for cell survival, cytokine gene induction, and cell cycle progression in T cells (17).

Although an essential role for PI3K signaling in lymphocyte activation is indicated, it remains unknown how TCR activates PI3K. A number of studies have shown possible mechanisms: binding to phosphotyrosines in TRIM, LAT, SLP-76, or ZAP-70 through the p85 SH2 domain (15, 18–20); association with Src kinases by the p85 SH3 domain (21, 22); or direct activation of the p110 subunit by Ras (23–25). In this study, we describe the role of two adaptor molecules, SLP-76 and LAT, in the activation of PI3K signaling following TCR stimulation. We show that SLP-76...
associates with p85 in Jurkat T cells after the stimulation of TCR. We also investigated whether the association of these two molecules plays a role in the activation of PI3K by examining membrane localization and phosphorylation of Akt. Furthermore, we directly demonstrate the role of SLP-76 on the activation of PI3K/Akt by performing reconstitution experiments in SLP-76–deficient (J14) or LAT-deficient (JCam2) Jurkat T cells with wild type (Wt), tyrosine mutants, and membrane-targeted constructs of SLP-76. Results from the study not only show that TCR-induced PI3K/Akt activation is dependent on membrane translocation and tyrosine phosphorylation of SLP-76, but that LAT is also required for the localization of SLP-76 to the membrane. Collectively, these results suggest distinct roles of two adaptor molecules in the activation of PI3K signaling pathway following TCR ligation: SLP-76 for membrane translocation of p85 and LAT for membrane translocation of SLP-76.

Materials and Methods

Abs and reagents

Anti-human CD3 mAb (UCHT1) and anti-human CD28 mAb (CD28.2) from BD Pharmingen (San Diego, CA) was used for cell stimulation. For immunoprecipitation (IP) and immunoblotting (IB), we used anti-PI3K p85 and anti-LAT polyclonal Abs and anti-tyr (9E10) and anti-phosphotyrosine (4G10) mAbs from Upstate Biotechnology (Lake Placid, NY), anti-SLP-76 and anti-Akt polyclonal Abs from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-flag mAb (M2) from Sigma-Aldrich (St Louis, MO). Abs against phospho-Akt (pAkt, Ser473 and Thr 308), phospho-SLP-76 (pGSK3, Ser2), and phospho-ERK (pERK, Ser25,286), were from Cell Signaling Technology (Beverly, MA). Secondary Abs, anti-mouse IgG and anti-rabbit IgG conjugated with HRP, were purchased from Bio-Rad ( Hercules, CA). CellTracker Orange CMTMR and superantigen staphylococcal enterotoxin E (SEE) were purchased from Molecular Probes ( Eugene, OR) and Toxin Technology ( Sarasota, FL). Anti-human CD3 mAb, ECL Plus and ECL reagents were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). A Pan T cell isolation kit II from Miltenyi Biotec ( Bergisch Gladbach, Germany) was used for depletion of non-T cells (negative selection). An Amaxa human T cell Nucleofector kit was obtained from Lonza (Cologne, Germany). ON-TARGET plus SMARTpool small interfering RNA (siRNA) targeting human SLP-76 and LAT, and ON-TARGET plus non-targeting pool were purchased from Thermo Scientific (Cologne, Germany). ON-TARGET plus SMARTpool small interfering RNA (siRNA) targeting human SLP-76 and LAT, and ON-TARGET plus non-targeting pool were purchased from Thermo Scientific Dharmacon (Chicago, IL).

Plasmids

The plasmids carrying Wt SLP-76 and its various tyrosine mutants cloned into a modified pEF-Bos vector with an N-terminal sequence encoding the flag epitope were provided by Dr. G.A. Koretzky (University of Pennsylvania, Philadelphia, PA) (2–4). SLP-76 Wt or Gads binding domain mutant (SLP-G2) fused with the first 35 aa (transmembrane region [TM]) of LAT in MigR1 and SLP-G2 in pEGFP were also provided by Dr. G.A. Koretzky (26). The plasmid expressing the LAT-TM fusion of three tyrosine mutants (TYF) of SLP-76 was generated by ligating PCR fragments of the LAT-TM flanked by BamHl/BglII sites and of the SLP-76-TYF flanked by BamHl/EcoRI into MigR1 vector (a gift from W. Pear, University of Pennsylvania, through G.A. Koretzky) (27) at the BglII/EcoRI sites. LAT Wt c-DNA was obtained from Jurkat T RNA by RT-PCR and subcloned into MigR1 vector by RT-PCR. The resulting plasmid was thereafter confirmed by sequencing. GFP-Akt-pleckstrin homology (PH) was a gift from Dr. T. Hunter.

Expression of plasmid DNA

Jurkat, JCam2, and J14 cells were electroporated using a Gene Pulser (Bio-Rad) at a setting of 250 V and 950 microfarad or a BTX-B280 Electro-SquarePiorator (Genetronics, San Diego, CA) at a setting of 220 V and 65 ms in cuvettes containing 2 × 106 cells in 0.4 ml Cytoxix (31) intracellular buffer (pH 7.6: 120 mM KCl, 0.15 mM CaCl2, 10 mM K2HPO4, 25 mM HEPES, 2 mM EGTA, and 5 mM MgCl2) and 20–40 μg indicated plasmid DNA. For transient expression of plasmid DNA, cells were electroporated in growth medium and incubated in growth medium for 16 h before transfection. For stable cell generation, cells were divided into seven petri dishes, and 10 days later, cells were sorted for expression of GFP using a FACSaria (BD Biosciences, Mountain View, CA). After an additional 2 wk in culture, cells were sorted again to obtain stable transfectants showing GFP fluorescence. Similar levels of surface TCR expression were confirmed. The cell lines were monitored regularly for expression of transfected proteins.

Human T cell purification and nucleofection

Samples of peripheral blood were collected from normal healthy donors. Written informed consent was obtained from all donors, and all studies were approved by the Institutional Human Ethics Review Board of Ewha Womans University Medical Center. Mononuclear cells were isolated by Ficoll-Paque Plus density centrifugation, washed, and suspended in PBS containing 0.5% BSA. The T cell fraction at 7 d in culture was depleted of non-T cells with superparamagnetic microbead selection using a mixture of mAbs and MiniMACS columns (Miltenyi Biotec). The efficiency of purification was verified by flow cytometry, counterstaining with R-PE-antihuman CD3 mAb, and usually reached the range of 94–99%. Nucleofection was performed using an Amaxa human T cell Nucleofector kit (Lonza, Cologne, Germany) following the manufacturer’s optimized protocol for unstimulated human T cells. Briefly, purified human T cells (4 × 106 cells/sample) were washed and the cell pellet was carefully resuspended in 100 μl Nucleofector solution. After combining 100 μl cell suspension simultaneously with 5 μg indicated plasmid DNA and 300 nM indicated siRNA, the suspension was transferred into a certified cuvette and the selected program (U-014) of the Amaxa Nucleofector kit was applied. The cells were cultured in growth medium for 48 h before performing experiments. For activation, the cells were washed and resuspended in RPMI 1640 medium at 1 × 106 cells/ml and incubated with either isotype control Ab or anti-CD3 Ab (5 μg/ml) for 5 min at 37°C.

IP and IB

T cell stimulation was terminated by adding an equal volume of ice-cold medium, and cell lysates were prepared in 1% Nonidet P-40 lysis buffer containing protease inhibitors (50 μg/ml aprotinin, 10 μg/ml leupeptin, 40 μg/ml pepstatin A, and 1 mM PMSF) and phosphatase inhibitors (400 μM sodium vanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate), as described previously (32). For IB, lysates from 1 × 106 cells were mixed with 2× Laemmli sample buffer, boiled, and subjected to 10% SDS-PAGE. After transferring proteins to a nitrocellulose membrane, IB was performed by blocking the membrane with 5% nonfat dried milk and incubating with various primary Abs, followed by HRP-conjugated secondary Abs. Detection was conducted with ECL reagents. For IB, lysates from 1 × 106 cells were tumbled with GammaBind G-Sepharose beads conjugated with individual Abs as indicated. The immune complexes were then subjected to SDS-PAGE, followed by IB.

Isolation of GEM fractions

GEM fractions were isolated as described previously with slight modifications (33). Briefly, MNCs prepared from Jurkat T cells (1 × 108) were washed twice in PB, resuspended in 900 μl buffer A (25 mM HEPES, 150 mM NaCl, and 1 mM EGTA, with protease and phosphatase inhibitors described for the Nonidet P-40 lysis buffer), and preincubated for 4 min at 37°C. The detergent Brij-98 in buffer A (100 μl) was added to achieve a final concentration of 1%, and the cell suspensions were further incubated for 5 min at 37°C. Cell lysates were then mixed with 1 ml 80% sucrose and prewarmed buffer A and immediately chilled on ice for 55 min. After transfer to an ultracentrifuge tube, the samples were overlaid with 6.5 ml 30% sucrose in buffer B, followed by 1 ml 5% sucrose in buffer A. All procedures were performed on ice. The Brij-insoluble fractions were

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separated from the cell lysates by ultracentrifugation for 18 h at 40,000 rpm in a Beckman SW 40.1 Ti rotor at 4°C. Fractions (1 ml) were collected sequentially from the top of the gradient and analyzed by IB.

**Immunolabeling for membrane and immunofluorescence**

For conjugation of Jurkat T cells, Raji B cells were used as APCs (34). Prior to conjugation, Raji B cells were stained with CMTMR (4 μM) for 5 min at 37°C, washed, and incubated in RPMI 1640 medium with or without SEE (5 μg/ml) for 1 h at 37°C. CMTMR-stained Raji B cells and Jurkat T cells that were transfected with expression plasmids encoding GFP-Akt-3P or an empty vector were mixed at a 1:1 ratio, immediately transferred to poly-L-lysine-coated slides, and incubated for 15 min at 37°C. Slides were fixed in 3.7% formaldehyde for 20 min at room temperature and analyzed using a Zeiss Axiovert 200 fluorescence microscope that was equipped with a ×40 objective lens (Carl Zeiss, Oberkochen, Germany). Background fluorescence levels in T cells that express vector-associated GFP were minimal. The settings of the microscopy that corrected for any background fluorescence in T cells were used for fluorescence measurements. All images were acquired and analyzed with Zeiss software. Images were further processed and quantified with Wright Cell Imaging Facility ImageJ.

**Results**

**Association of p85 with tyrosine-phosphorylated SLP-76 results in the phosphorylation of Akt following TCR stimulation**

We previously reported that SLP-76 associates with p85, a subunit of PI3K, in Jurkat T cells after the activation of TCR (15). To examine the role of this association in the activation of PI3K signaling, we assayed phosphorylation of Akt, an event that occurs downstream of PI3K. We first measured the association of p85 with tyrosine-phosphorylated SLP-76 in TCR-stimulated Jurkat cells or a Jurkat variant cell line that lacks SLP-76 or LAT (Fig. 1A). Reciprocal IP detected the association of SLP-76 with p85 only in Jurkat T cells in which tyrosine phosphorylation of SLP-76 was induced following TCR stimulation (Fig. 1A). To detect the activation of PI3K signaling in Jurkat or Jurkat variant cells, phosphorylation of Akt was measured in these cells following TCR stimulation. To enhance the sensitivity of detection of Akt phosphorylation, especially in cells transfected with various DNA plasmids throughout the study, myc-tagged Akt was expressed and phosphorylation was analyzed by anti-myc IP. As shown in Fig. 1B, both endogenous and transfected myc-tagged Akt was phosphorylated in Jurkat T cells, but not in SLP-76 or LAT-deficient mutants, J14 or JCam2 cells, after TCR activation. These results show a correlation between tyrosine-phosphorylated SLP-76 association with p85 and PI3K activation in TCR-stimulated T cells.

Because Jurkat T cells are deficient in PTEN (35), we next examined the phosphorylation of Akt in Jurkat T cells supplemented with a WT PTEN plasmid. As shown in Fig. 2A, the phosphorylation of Akt (both Ser^473 and Thr^308) was similar in Jurkat and PTEN-transfected Jurkat T cells after TCR activation. Furthermore, because the binding site for PI3K lies within the YNNM motif of the CD28 cytoplasmic domain (36), we analyzed the phosphorylation of Akt in Jurkat T cells after CD28 costimulation. Similar levels of Akt phosphorylation were detected after TCR stimulation alone or together with CD28 costimulation, but a slight phosphorylation of Akt was seen after CD28 stimulation alone (Fig. 2B).

Tyrosine phosphorylation of SLP-76 at position 113 and/or 128 is required for association with p85 and consequent phosphorylation of Akt after TCR activation

Previously, we also demonstrated that tyrosine phosphorylation at residue 113 and/or 128 is sufficient for the association of SLP-76 with the SH2 domain near the N terminus of p85 (15). In this study, we examined the role of these SLP-76 tyrosines in the activation of PI3K signaling. J14 cells were reconstituted with Wt or various tyrosine mutants of SLP-76 along with myc-tagged Akt, and TCR-

![FIGURE 1.](image-url) Association of tyrosine-phosphorylated SLP-76 with p85 leads to Akt phosphorylation following TCR activation. A, Jurkat, SLP-76^−/−, and LAT^−/− cells were either unstimulated (−) or TCR stimulated (+) for 5 min at 37°C. Lysates were immunoprecipitated with Ab against either SLP-76 or p85. Immunoprecipitates were divided equally and both sets were subjected to SDS-10% PAGE. For IP with anti-SLP-76, tyrosine phosphorylation of SLP-76 following TCR ligation was detected by IB with 4G10, and the same blot was reprobed with anti-SLP-76 for loading controls for IP reactions. To measure phosphorylation-dependent association of SLP-76 with p85, another blot was detected by IB with anti-p85 Ab. For IP with anti-p85, tyrosine phosphorylation of SLP-76 following TCR ligation was detected by IB with 4G10, and the same blot was reprobed with anti-SLP-76 to measure phosphorylation-dependent association of SLP-76 with p85. For loading controls for IP reactions, another blot was detected by IB with anti-p85 Ab. Data are representative of three independent experiments. B, Jurkat, SLP-76^−/−, and LAT^−/− cells were transfected with a plasmid carrying Akt (myc). The cells were either left unstimulated (−) or TCR stimulated (+) for 5 min at 37°C, and lysates were prepared and subjected to SDS-10% PAGE. Protein expression was detected by IB with anti-myc Ab. Activation induced by TCR stimulation was determined by IB with anti-pAkt Ab. Immunoprecipitates with anti-myc Ab, as well as control anti-mouse Ig (data not shown), were subjected to SDS-10% PAGE. The amounts of IP from unstimulated or TCR stimulated lysates were determined by IB with anti-myc Ab and phosphorylation of Akt, both endogenous (endo) and transfected (myc), following TCR stimulation was confirmed by IB with anti-pAkt Ab. Data shown are representative of three separate experiments.
induced phosphorylation of Akt was detected by blotting with anti-myc Ab (Fig. 3A). Consistent with results of the previous interaction studies of SLP-76 with p85, Akt phosphorylation following TCR stimulation was diminished in J14 cells reconstituted with the SLP-76-Y113F mutant, but not with SLP-76-Y128F or SLP-76-Y145F (Fig. 3A). Akt phosphorylation following TCR stimulation was further diminished in J14 cells reconstituted with the double tyrosine mutant (Y113, 128F) or triple tyrosine mutant (TYF) (Fig. 3A). The normalization of pAkt to the level of anti-myc IP, followed by calculation of induced phosphorylation of Akt after TCR activation, is shown in Fig. 3B. These results suggest that the association of SLP-76 with p85 through phosphorylated tyrosines after TCR stimulation correlates with the activation of PI3K signaling.

Tyrosine phosphorylation of SLP-76 in the absence of LAT is not sufficient for Akt phosphorylation following TCR activation

To determine whether tyrosine phosphorylation of SLP-76 is sufficient for Akt phosphorylation, we compared the expression of WT SLP-76 with the phosphorylation of Akt following TCR stimulation in Jurkat, J14, and JCam2 cells. As shown in Fig. 4A, the levels of TCR-stimulated phosphorylation of Akt were similar

FIGURE 2. Akt phosphorylation in Jurkat T cells after reconstitution with PTEN followed by TCR activation, or after costimulation with TCR and CD28. A, Jurkat T cells were transfected with empty vector (−) or PTEN Wt plasmid [HA (+)]. The cells were either unstimulated (−) or TCR stimulated (+) for 5 min at 37˚C, and lysates were prepared and subjected to SDS-10% PAGE. PTEN expression was detected by IB with anti-HA Ab. Activation induced by TCR stimulation was determined by IB with anti-pAkt Ab (both Ser473 and Thr308). For loading controls in each lane, the same blot probed with anti-pAkt Ab (Thr308) was reprobed with anti-Akt Ab. Data are representative of three independent experiments. B, Jurkat cells were either unstimulated (−), or costimulated with TCR+ or CD28+, alone, or costimulated with TCR+ and CD28+ for 5 min at 37˚C. Lysates were prepared and subjected to SDS-10% PAGE. Activation induced by TCR stimulation was determined by IB with anti-pAkt Ab (both Ser473 and Thr308). For loading controls in each lane, the same blot probed with anti-pAkt Ab (Thr308) was reprobed with anti-Akt Ab. Data are representative of three independent experiments.

FIGURE 3. Phosphorylation of SLP-76 at tyrosines 113 and/or 128 is required for Akt phosphorylation following TCR activation. A, J14 cells were reconstituted with plasmids carrying SLP-76 Wt (flag) or various tyrosine substitution mutants (flag) along with Akt (myc). The cells were either left unstimulated (−) or TCR stimulated (+) for 5 min at 37˚C, and lysates were prepared and subjected to SDS-10% PAGE. Protein expression was detected by IB with anti-flag or anti-myc Ab. Immunoprecipitates with anti-myc Ab, as well as control anti-mouse Ig (data not shown), were subjected to SDS-10% PAGE. The amounts of IP from lysates were determined by IB with anti-myc Ab, and phosphorylation of Akt following TCR stimulation was confirmed by IB with anti-pAkt Ab. Representative data from three independent experiments are shown. B, The intensities of the bands in pAkt and myc blots were quantified and the normalization of pAkt to the level of anti-myc IP was followed. Values indicate the intensity after TCR stimulation relative to the unstimulated control.
in Jurkat T cells overexpressing Wt SLP-76, J14 cells reconstituted with Wt SLP-76, and Jurkat cells transfected with a vector control. In contrast, overexpression of Wt SLP-76 did not induce phosphorylation of Akt after TCR stimulation in JCam2 cells (Fig. 4A). These results clearly demonstrate that overexpression of SLP-76 in the absence of LAT is not sufficient to cause the phosphorylation of Akt following TCR stimulation. Moreover, overexpression of tyrosine mutant SLP-76 (TYF) in Jurkat cells did not affect TCR-induced phosphorylation of Akt in Jurkat cells, as shown in Fig. 4B. These results suggest that overexpression of the SLP-76 triple tyrosine mutant did not interfere with the interaction between the endogenous tyrosine-phosphorylated SLP-76 and p85, and that the interaction is mediated only by the phosphorylation of SLP-76. Fig. 4B also shows the results of overexpressing SLP-76 (TYF) in J14 and JCam2 cells. The fact that reconstitution of J14 cells with SLP-76 (TYF) did not induce phosphorylation of Akt following TCR stimulation further demonstrates that the association of SLP-76 with p85 through its phosphorylated tyrosines is required for TCR-induced activation of the PI3K cascade.

**GEM recruitment of p85 and immunological synapse localization of Akt are fully induced by TCR activation in the presence of both SLP-76 and LAT**

We next examined the membrane translocation of both p85 and SLP-76 and the consequent Akt recruitment to immunological synapse (IS) after TCR activation. As expected, translocation of both SLP-76 and p85 to GEMs following TCR stimulation occurred only in Jurkat T cells, and not in J14 and JCam2 cells, as determined by IB analysis of GEM fractions identified by the presence of LAT and GM1 (Fig. 5A). The IS formed between T cells and APCs in the presence and absence of SEE, as well as the subsequent recruitment of Akt in T cells, is shown in Fig. 5B. Maximum fluorescence intensity by Akt recruitment to the IS in each T cell–APC conjugate in the presence of SEE was quantified and the intensity values of 35 conjugates were compared between Jurkat, J14, and JCam2 cells, as shown in Fig. 5C. The GFP-tagged PH domain of Akt (GFP-Akt-PH) was fully recruited to IS, where phospholipid second messengers are generated by activated PI3K in Jurkat cells. In contrast, partial or no recruitment of Akt-PH to IS was observed in J14 or JCam2 cells (Fig. 5B, 5C).

**Membrane-targeted SLP-76 is sufficient for the activation of PI3K signaling following TCR ligation**

The association of p85 with tyrosine-phosphorylated SLP-76 or LAT following TCR activation has been demonstrated previously (15, 18, 37). However, p85 association with either SLP-76 or LAT alone did not lead to the activation of PI3K, as shown in Figs. 4 and 5. To investigate the roles of the two adaptor proteins, SLP-76 and LAT, in PI3K activation, we performed reconstitution experiments with various constructs of SLP-76 and LAT in SLP-76-deficient J14 or LAT-deficient JCam2 cells.

As shown in Fig. 6A, following stimulation of TCR alone or together with CD28, the PI3K signaling pathway was activated in Jurkat cells, but not in J14 and JCam2 cells, as demonstrated by the phosphorylation of downstream signaling molecules Akt, Gsk3β, and FKHR. To examine whether SLP-76 association with LAT or membrane targeting of SLP-76 is required for the PI3K signaling after TCR ligation, SLP-76-deficient J14 cells were reconstituted with SLP-G2 (Gads binding domain mutant of SLP-76; consequently not associated with LAT) or TM-SLP-G2 (SLP-G2 fused to LAT TM; consequently membrane-targeted). Expression of SLP-76 Wt and TM-SLP-G2 constructs, but not the SLP-G2 construct alone, reconstituted the TCR- and TCR/CD28-induced PI3K signaling pathways in J14 cells (Fig. 6B). Moreover, transformation of J14 cells with membrane-targeted SLP-76 (TM-SLP-Wt), but not with membrane-targeted tyrosine mutants of SLP-76 (TM-SLP-TYF), induced PI3K signaling following stimulation with TCR alone or together with CD28 (Fig. 6C). Expression of LAT Wt and TM-SLP-Wt constructs, but not the TM-SLP-TYF construct, also reconstituted the TCR- and TCR/CD28-induced PI3K signaling pathways in JCam2 cells (Fig. 6D). These results demonstrate that both events, that is, tyrosine phosphorylation of SLP-76 and membrane targeting of SLP-76 by LAT, are required for the activation of PI3K through T cell activation.

The translocation of p85 to GEMs following TCR stimulation was also measured by IB analysis of GEM fractions in J14 cells reconstituted with the TM-SLP-G2 construct and in JCam2 cells reconstituted with TM-SLP-Wt (Fig. 7A). In contrast, the GEM recruitment of p85 was not induced by TCR activation in J14 cells reconstituted with the SLP-G2 construct or in JCam2 cells reconstituted with the TM-SLP-TYF construct (Fig. 7A). In this study, membrane-targeted SLP-76 proteins (TM-SLP-G2, TM-SLP-Wt, and TM-SLP-TYF) were also shown in GEM fractions, but SLP-G2 was not translocated to GEMs following TCR stimulation due to lack of association with LAT (Fig. 7A). Similarly, resident Wt SLP-76 in LAT-deficient JCam2 cells was not identified in GEMs when probed with anti-STP-76, because the recruitment of SLP-76 to GEMs occurs through its indirect association with LAT by Gads binding (Fig. 7A).

The subsequent recruitment of Akt to the IS formed with APCs in the presence and absence of SEE was detected by fluorescence detection of the transfected GFP-Akt-PH (Fig. 7B). As shown in Fig. 5C, the fluorescence intensities representing Akt recruitment...
to the IS in T cell–APC conjugates in the presence of SEE are compared in Fig. 7C. The IS localization of Akt was induced by TCR activation in J14 cells reconstituted with SLP-Wt (Fig. 7B, 7C). However, recruitment of Akt to the IS was not detected in J14 cells reconstituted with the SLP-G2 construct (Fig. 7B, 7C). These results, together with those of p85 recruitment to the GEM, suggest that the induction of TCR-activated PI3K signaling requires the recruitment of SLP-76 to the GEM through an indirect association with LAT by Gads binding and the subsequent recruitment of Akt to the IS formed with APCs.

Similarly, the IS localization of Akt was induced by TCR activation in JCam2 cells reconstituted with TM-SLP-Wt (Fig. 7B, 7C). However, recruitment of Akt to the IS following TCR stimulation was not observed in JCam2 cells reconstituted with the TM-SLP-TYF construct (Fig. 7B, 7C). These results further suggest that membrane-targeted tyrosine-phosphorylated SLP-76 is sufficient for the induction of TCR-activated PI3K signaling.

Tyrosine phosphorylation of SLP-76 and its membrane translocation by LAT are also required for TCR-induced PI3K activation in primary T cells isolated from human peripheral blood

From experiments performed using various Jurkat T cell systems, we conclude that SLP-76 and LAT are both required for translocation of p85 to the membrane and subsequent phosphorylation of Akt, and that the two adaptor proteins have different functions in the process. SLP-76 associates with p85 after T cell activation, and then LAT recruits this complex to the membrane. To verify these findings in primary T cell systems, we carried out key experiments in primary T cells isolated from human peripheral blood.

Human T cells were electroporated with a scrambled siRNA or a specific siRNA for either SLP-76 or the LAT gene. Human T cells, in which each endogenous gene was suppressed, were also electroporated with a TM-SLP-Wt or TM-SLP-TYF plasmid. As shown in Fig. 8A and 8C, endogenous SLP-76 or LAT was suppressed by specific siRNA, but not by scrambled siRNA. Exogenously transfected plasmids, TM-SLP-Wt and TM-SLP-TYF, were also expressed in human T cells in which each gene was suppressed (Fig. 8A, 8C).

Following stimulation with TCR, the PI3K signaling pathway was activated in cells transfected with a scrambled siRNA, but not with siRNA specific for either SLP-76 or LAT, as demonstrated by the phosphorylation of downstream signaling molecules Akt, Gsk3β, and FKHR (Fig. 8B, 8D). Expression of TM-SLP-Wt, but not the TM-SLP-TYF construct, reconstituted TCR-induced PI3K signaling pathways in cells that had either the SLP-76 or LAT gene suppressed (Fig. 8B, 8D). We also obtained the same results from the same experiments performed in primary T cells isolated from mouse spleen (data not shown). The results from experiments in primary T cells further demonstrate that tyrosine phosphorylation of SLP-76 and membrane targeting of SLP-76 by LAT are both required for the activation of PI3K following TCR stimulation.

Discussion

In this report, we describe the role of two adaptor molecules, SLP-76 and LAT, in the activation of PI3K signaling following TCR stimulation. Both SLP-76 and LAT are substrates of ZAP-70/Syk protein tyrosine kinases and are known to associate with several SH2 domain-containing molecules, including the p85 subunit of PI3K, through phosphotyrosine residues upon T cell activation (15, 18, 37).

Upon T cell activation, LAT associates directly with Grb2, phospholipase Cγ1, and the p85 subunit of PI3K. Two tyrosine
residues, Y171 and Y191 of LAT, appear to be required for association with these signaling molecules (38). LAT binds Cbl, Sos, Vav, and SLP-76, probably indirectly via Grb2 (38). SLP-76 associates with Vav1, noncatalytic kinase, IL-2–induced tyrosine kinase, and the p85 subunit of PI3K through phosphorylation of tyrosine residues Y113, Y128, and Y145 upon T cell activation (15, 39). Upon TCR activation, the tyrosine phosphorylation and consequent association of these proteins occur at a special junction, the IS, formed at the T cell–APC interface (40, 41).

The family of PI3Ks includes the p85/p110 isoform, which comprises two associated proteins, the p85 regulatory subunit and the catalytic subunit p110 (42–45). p85 contains two SH2 domains and one SH3 domain, which allow interactions with other signaling proteins, resulting in membrane translocation and activation of the p110 catalytic subunit. p85 is recruited to the plasma membrane through its SH2 domain by tyrosine-phosphorylated adaptor molecules, and then 3’-phosphoinositides are produced by the activated PI3K catalytic subunit. The production of PI3P and PI3P3, which are undetectable in unstimulated cells, is a key event during signaling, as it allows the membrane recruitment of many PH domain-containing proteins involved in cell growth, survival, or cytoskeletal rearrangement (43, 44).

In T cells, Ag recognition is followed by rapid and sustained accumulation of the PI3K product, PI3P3 at the plasma membrane, particularly concentrated at the IS (36, 45, 46). Genetic manipulations that enhance PI3K pathway activity cause lymphoproliferation in mice (47–49). Conversely, pharmacologic inhibitors of PI3K, such as wortmannin and LY294002, potently block T cell proliferation (50, 51). These observations are indicative of an essential role for PI3K signaling in lymphocyte activation. How TCR activates PI3K remains unknown, although a number of possible mechanisms have been reported: binding to YXXM motifs in the transmembrane adaptor protein TRIM or to non-canonical phosphotyrosines in LAT, SLP-76, or ZAP-70 through the p85 SH2 domain (15, 18–20); association with Src kinases by the p85 SH3 domain (21, 22); or direct activation of p110 subunit by Ras (23–25).

The clearest link between T lymphocyte signaling and PI3K activation is the costimulatory molecule CD28. The p85 regulatory subunit of PI3K, being associated with a phosphorylated YXXM motif in the cytoplasmic tail of CD28, recruits the p110 catalytic subunit, which converts PI3P2 to PI3P3 at the membrane (36, 52, 53). Locally generated PI3P3 serves as a docking site for the PH domain of PDK1 and its target Akt, resulting in the sequential activation of these kinases. Activated Akt phosphorylates multiple proteins, including Gsk3β and FKHR, enabling them to affect numerous cellular responses (36, 52, 53).

In this report, we studied the role of two adaptor molecules, SLP-76 and LAT, in PI3K activation following TCR ligation. It has been shown that both SLP-76 and LAT associate with the SH2 domain of p85 through their phosphotyrosine residues upon TCR activation (15, 18, 37). In this study, we demonstrated the TCR-induced interaction of p85 and SLP-76 occurs in Jurkat T cells, but not in J14 or JCam2 cells, and results in Akt activation upon T cell activation (Fig. 1). Reconstitution of J14 cells with various SLP-76 mutants demonstrated that tyrosine phosphorylation at 113 or 128 is sufficient for the activation of Akt (Fig. 3). Additionally, we show that membrane-targeted forms of Wt SLP-76, but not of its tyrosine mutants, reconstituted Akt phosphorylation in activated cells. Stimulation of all cells and analyses of activation were followed, as described in A. Representative data from three independent experiments are shown.
it varied greatly with experimental conditions, such as resting condition of the cells. A similar level of endogenous phosphorylation of Akt was also observed in primary human T cell experiments. Above all, we did not see much of a difference in the level of Akt activation after PTEN was added back to Jurkat T cells (Fig. 2A).

FIGURE 7. Recruitment of p85 and Akt to GEMs and IS following TCR stimulation in SLP-76<sup>−</sup> or LAT<sup>−</sup> cells reconstituted with TM-SLP-G2 or TM-SLP-Wt, but not with SLP-G2 or TM-SLP-TYF plasmid. A, SLP-76<sup>−</sup> cells stably reconstituted with TM-SLP-G2 or SLP-G2 plasmid (SLP-76<sup>−</sup>) and LAT<sup>−</sup> cells stably reconstituted with TM-SLP-Wt or SLP-TYF plasmid (LAT<sup>−</sup>) were stimulated with either control or anti-CD3 Abs for 5 min at 37˚C. GEM fractions were isolated from these cells by density gradient ultracentrifugation, and IB was performed with anti-p85 and anti-SLP-76 Abs, as described for Fig. 5A. Data are representative of three separate experiments. B and C, SLP-76<sup>−</sup>/TM-SLP-G2 or SLP-G2, and LAT<sup>−</sup>/TM-SLP-Wt or TM-SLP-TYF cells were transfected with the GFP-Akt-PH plasmid. Cell stimulation and IS localization analysis of GFP-Akt-PH (arrowhead) were followed, as described for Fig. 5B. Representative fields of the merged fluorescence pattern for each group are shown from three separate experiments. *p < 0.05, one-way ANOVA (Tukey multiple comparison test).

FIGURE 8. Expression of membrane-targeted and tyrosine-phosphorylated SLP-76 activates PI3K signaling pathway after TCR stimulation in primary human T cells in which the SLP-76 or LAT gene was suppressed. A and C, Primary T cells isolated from human peripheral blood were transfected with scrambled or SLP-76 (or LAT) gene-specific siRNA. Either TM-SLP-Wt or TM-SLP-TYF plasmid was expressed in SLP-76 (or LAT) gene-suppressed primary human T cells. Cell lysates were prepared and subjected to SDS-10% PAGE. Gene suppression, as well as expression of both endogenous (endo) and exogenously transfected (exo) SLP-76, was detected by IB with anti-SLP-76 and anti-LAT Abs. B and D, The cells prepared in A and C were either unstimulated (−) or TCR stimulated (+) for 5 min at 37˚C, and lysates were prepared and subjected to SDS-10% PAGE. Phosphorylation of proteins following stimulation was detected by IB with indicated phospho-protein–specific Abs. The same blot probed with anti-pAkt was reprobed with anti-Akt Ab to demonstrate equal sample loading. All experiments were repeated twice and similar results were obtained.
CD28 costimulation also does not seem to enhance TCR-induced PI3K activation in our Jurkat system. As shown in Fig. 2B, stimulation of CD28 alone induced a small amount of Akt activation, whereas costimulation of TCR/CD28 did not significantly enhance the TCR-induced Akt activation detected by phosphorylation of either Ser^{473} or Thr^{308}. These results are somewhat contradictory to the results obtained in primary mouse T cells, where CD28 stimulation and TCR/CD28 costimulation strongly induced Akt activation compared with TCR stimulation alone (55). However, in the same report, the level of Akt activation by TCR or CD28 stimulation or TCR/CD28 costimulation of Jurkat T cells was similar to what we measured (55). This variability could be related to differences in the degree of surface receptor cross-linking by different Abs or a difference between the origin and maintenance of T cells used.

Three tyrosine residues in the adaptor protein SLP-76 reside within YE(S/P)P motif: both Y113 and Y128 within YESP, and Y145 within YEPP (3). These residues are phosphorylated upon receptor ligation and associate with many signaling molecules that contain SH2 domains (39, 56). Association of Y113 and Y128 with Vav and noncatalytic kinase is important for TCR-induced phosphorylation of proximal signaling substrates (39, 56). Y145 is important for the optimal association of SLP-76 with IL-2–induced tyrosine kinase, and it is the most critical tyrosine for T cell development and functions (56, 57). We have previously shown that SLP-76 associates with the p85 subunit of PI3K and that phosphorylation of Y113 or Y128 is sufficient for binding to p85 (15). TCR-induced Akt phosphorylation was inhibited in SLP-76–deficient J14 cells reconstituted with mutant SLP-76 with Y113F, Y113F, Y113F, and Y128F, or YTF (Y113, 128, and 145F), and this result further demonstrates SLP-76 involvement in the PI3K signaling pathway (Fig. 3). However, it is possible that other molecules that associate with the same residues within SLP-76 are involved in PI3K activation. Vav, which associates with the Y113 residue of SLP-76 after TCR ligation, is one candidate because Vav also has a role in PI3K activation (58, 59).

LAT undergoes a post-translational modification in the form of palmitoylation, which leads to its localization to GEM fractions, and this localization is essential for LAT signaling (12, 18, 38). Many signaling molecules, including p85, Vav, and SLP-76, are associated either directly or indirectly with LAT after TCR activation, and they also localize to GEM (18, 38). In this study, we show that TCR-induced localization of p85 and SLP-76 did not occur at the GEM of LAT-deficient JCam2 cells (Fig. 5A). Consequently, Akt localization to IS of T cell–APC conjugates was completely lost in JCam2 cells, as shown in Fig. 5B and 5C. When Akt activation was measured by phosphorylation of Akt, we found that Akt phosphorylation at Ser^{473}, as well as Thr^{308} was undetectable after stimulation with TCR or TCR/CD28 in the absence of LAT (data not shown). Other studies have shown that PI3K is stably activated in the IS of T cells/APCs to stimulate Akt phosphorylation of the SLP-76 adaptor protein in either the Jurkat T cell system (Figs. 4, 6, 7) or primary human T cells (Fig. 8A, 8B). We also show that LAT is required for the localization of SLP-76 to the membrane in Jurkat cells (Figs. 4, 6, 7) and primary human T cells (Fig. 8C, 8D). These findings suggest a new paradigm for PI3K/Akt activation mechanisms that are known to be required for T cell activation. At the same time, this new mechanism of collaboration between SLP-76 and LAT adaptor molecules may provide a reason to re-evaluate many controversial results and issues regarding PI3K/Akt activation signaling in T lymphocytes, such as the Vav1 independence of the activation of Akt (55). Collectively, the results of this study illustrate distinct roles for two adaptor molecules, SLP-76 and LAT, in the activation of PI3K following TCR ligation.

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