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*J Immunol* 2006; 176:2430-2438; doi: 10.4049/jimmunol.176.4.2430
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Functional Hierarchy of the N-Terminal Tyrosines of SLP-76

Martha S. Jordan,*† Jeffrey Sadler,* Jessica E. Austin,* Lisa D. Finkelstein,‡ Andrew L. Singer,* Pamela L. Schwartzberg,‡ and Gary A. Koretzky2*§

The adaptor protein Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) plays a central role in T cell activation and T cell development. SLP-76 has three functional modules: an acidic domain with three key tyrosines, a central proline-rich domain, and a C-terminal Src homology 2 domain. Of these, mutation of the three N-terminal tyrosines (Y112, Y128, and Y145) results in the most profound effects on T cell development and function. Y112 and Y128 associate with Vav and Nck, two proteins shown to be important for TCR-induced phosphorylation of proximal signaling substrates, Ca2+ flux, and actin reorganization. Y145 has been shown to be important for optimal association of SLP-76 with inducible tyrosine kinase, a key regulator of T cell function. To investigate further the role of the phosphorylatable tyrosines of SLP-76 in TCR signaling, cell lines and primary T cells expressing SLP-76 with mutations in individual or paired tyrosine residues were analyzed. These studies show that Tyr145 of SLP-76 is the most critical tyrosine for both T cell function in vitro and T cell development in vivo. The Journal of Immunology, 2006, 176: 2430–2438.

Signaling through the TCR requires the coordinated assembly of multiple proteins. Src homology (SH)2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) is an adaptor that acts as a molecular scaffold to insure that this assembly occurs in the correct spatiotemporal manner (1, 2). The critical nature of SLP-76 has been demonstrated in cell lines and in vivo. In the Jurkat T cell derivative J14, which lacks SLP-76 expression, signaling through the TCR fails to result in phospholipase Cγ1 (PLCγ1) phosphorylation, Ca2+ mobilization, or the activation of TCR-dependent transcription factors such as NF-AT and AP-1 (3). In vivo, SLP-76 deficiency results in a complete arrest of T cell development at the CD4+CD8− double-negative (DN) stage (DN3) (4, 5).

SLP-76 has three domains, all of which are required for optimal SLP-76 function (6–8). The C terminus consists of an SH2 domain that inducibly associates with adhesion- and degranulation-promoting adaptor protein and hemopoietic progenitor kinase 1 (9–11). The central region of SLP-76 is proline rich and contains an RxxK motif that is responsible for its constitutive association with the adaptor Gads (12, 13). Gads inducibly associates with linker of activated T cells following T cell phosphorylation. It is through its association with Gads that SLP-76 is recruited to the plasma membrane, as mutation of this domain results in loss of proper SLP-76 localization and function (13, 14). Within the proline-rich region, an N-terminal stretch termed the P1 domain has been described. This sequence has been reported to be the site of constitutive PLCγ1 association (15). A small amino acid stretch distal to the acidic domain of SLP-76 has been reported to be responsible for binding to the Src family kinase Lck (16). The role of this interaction has yet to be defined.

Within the N terminus of SLP-76 are three tyrosines at positions 112 (113 in human), 128, and 145. Y112 and Y128 are part of two YESP motifs. Both of these tyrosine residues are required for binding to the guanine nucleotide exchange factor Vav, as mutation of either results in the loss of a SLP-76/Vav interaction (17–22). The adaptor Nck also associates with the YESP motifs of SLP-76 (19, 23). Phosphopeptide-blocking studies have suggested that this interaction is more dependent upon phosphorylation of Y128 (the second YESP motif) than Y112 (19, 23). The N-terminal tyrosines of SLP-76 have also been implicated in mediating an SH2 domain-dependent interaction with the Tec family tyrosine kinase inducible T cell kinase (Itk) (24, 25). Studies in which phosphopeptides were used to map which tyrosine(s) was responsible for this interaction showed that a phospho-Y145 peptide was better at blocking the SLP-76/Itk interaction than a phospho-Y112 peptide, whereas a Y128 peptide showed no effect (24). These data implicated Y145 as a key residue in the interaction between SLP-76 and Itk.

In cell lines, mutation of Y112, Y128, and Y145 (termed Y3F) results in greatly diminished to nearly absent activation of several downstream SLP-76-dependent targets (6, 15, 26). In vivo, transgenic reconstitution of SLP-76-deficient mice with a Y3F mutant of SLP-76 only partially relieves the DN3 to DN4 block seen in SLP-76−/− mice. These mice develop CD4−CD8+ double-positive thymocytes, but the size of their thymus is only one-tenth that of wild-type (WT) mice (7, 8). A few CD4−CD8+ and CD4+CD8+ single-positive (SP) thymocytes are produced, but the ratio of CD4SP to CD8SP cells is skewed toward CD8SP cells. Moreover, these SP cells have low levels of TCR and do not express markers associated with normal positive selection. The
peripheral T cells found in these mice are refractory to TCR stimulation; they do not phosphorylate key signaling proteins such as PLCγ1, mobilize Ca2+, or up-regulate activation markers following TCR ligation (7, 8). In comparison with SLP-76−/− mice reconstituted with SLP-76-containing mutations in the proline-rich region or SH2 domain, the Y3F-reconstituted mouse has a much more severe phenotype (7, 8). Thus, both in vitro and in vivo data clearly demonstrate that the N-terminal tyrosines of SLP-76 have the biggest impact on the ability of SLP-76 to transmit signals to downstream effectors that direct thymocyte development and T cell activation.

Given the importance of the three N-terminal tyrosines of SLP-76 in aggregate, we investigated how each contributes to SLP-76 function both individually and as pairs. Although a previous study suggested the importance of these tyrosines in T cell SLP-76 function both individually and as pairs. Although a previous study suggested the importance of these tyrosines in T cell downstream effectors that direct thymocyte development and T cell activation.

Western blotting
J14 cells were rested in nonserum-containing medium at 37°C for 30 min. Cells (1 × 10^6 per condition) were resuspended in PBS containing Ca^2+ and Mg^2+ and left unstimulated, or stimulated with C305 ascites for the indicated time periods with or without pervanadate (PV) (30). Cells were then pelleted and lysed in 1% Nonidet P-40 lysis buffer containing a 1/100 dilution of protease inhibitor mixture human optimized (Sigma-Aldrich), 1 mM PMFS, 400 μM sodium vanadate, 10 μM sodium fluoride, and 10 μM sodium pyrophosphate. Lysates were diluted in 10% SDS loading buffer, boiled, and subjected to SDS-PAGE. Fold induction was quantitated using NIH Image software. For primary cells, lymph node cells and splenocytes were purified using mouse T cell enrichment columns, according to the manufacturer’s directions (R&D Systems). Cells were stimulated for the indicated times in plates coated with 5 μg/ml anti-CD3 (2C11; BD Pharmingen). Cells were lysed directly in loading buffer and sheared through a 25-gauge needle. The following Abs were used for Western blotting: anti-SLP-76, anti-PLCγ1, and phospho-PLCγ1 (Upstate Biotechnology); anti-Erk 1,2 (Zymed Laboratories); anti-phospho-Erk (Cell Signaling Technology); and anti-phospho-Y145-SLP-76 (BioSource International).

Materials and Methods
Plasmid constructs and stable cell lines
A→T base pair mutations of SLP-76 murine cDNA in pBluescript were generated using Transfector Mutagenesis (BD Clontech). These mutations result in tyrosine to phenylalanine substitutions at aa 112, 128, and 145 of SLP-76. WT and mutant SLP-76 cDNAs were cloned, excised from pBluescript with BamHI digestion, and cloned into the BglII site of the murine stem cell virus-based internal ribosomal entry site, GFP retroviral vector (MIGR1) (27) (a gift from W. Pear, University of Pennsylvania, Philadelphia, PA). MIGR1 plasmids were linearized with XspI. J14 cells were electroporated with 50 μg of linearized plasmid using a Gene Pulser (Bio-Rad) and cultured overnight with a range of anti-CD3 (5, 1, 0.1, 0.01, or 0.005 μg/ml) (2C11; BD Pharmingen) or PMA (50 ng/ml) and ionomycin (1 μg/ml). Cultures were incubated at 37°C for 6 h, after which time cells were harvested and lysed. The cell lysate was added to an equal volume of 2X luciferase buffer, and immediately before analysis, luciferin specific for the NF-AT reporter luciferase was added. The luminescent signal was quantitated using a TR717 Luminometer (Tropix). The reaction was stopped and samples were read a second time immediately after the addition of Renilla-specific luciferin. NF-AT luciferase values were normalized for transfection efficiency using values from the Renilla-luciferin reaction and expressed as percentage of maximal stimulation (PMA/ionomycin).

Flow cytometry
Cells were harvested, washed, and stained with Abs in FACS buffer (PBS containing 2% FBS and 0.002% azide) for 30 min. Cells were then washed, resuspended in FACS buffer, and analyzed on a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software (Tree Star). Abs for flow cytometry against human and murine Ags were purchased from BD Pharmingen and were as follows: anti-CD4 PerCP, anti-CD8 allophycocyanin, anti-CD3 PE, anti-CD69 PE, and anti-CD25 PE. For intracellular SLP-76 staining, cells were first fixed and permeabilized using Cytofix and Cytoperm, according to manufacturer’s instructions (BD Pharmingen), and then stained with an anti-murine SLP-76 PE Ab (28).

Up-regulation of activation markers
J14 cells were cultured overnight with various dilutions (over a range from 1/1,000 to 1/20,000) of C305 ascites (a gift from A. Weiss, University of California, San Francisco, CA (29)) or PMA (10 ng/ml) and ionomycin (200 ng/ml). Cells were harvested and stained with anti-human CD69 antibody, as described above. Splenocytes (3 × 10^6 cells/ml) were cultured overnight with a range of anti-CD3 (5, 1, 0.1, 0.01, or 0.005 μg/ml) (2C11; BD Pharmingen) or PMA and ionomycin. Cells were harvested and stained with anti-CD8 allophycocyanin, anti-CD4 PerCP, and either anti-CD69 PE or anti-CD25 PE.

Luciferase assay
J14 cell lines were transiently transfected with 20 μg of the NF-AT firefly-luciferase reporter plasmid and 5 μg of a Renilla-luciferase plasmid to control for transfection efficiency (Dual-Luciferase Reporter Assay; Promega). Following overnight culture, cells were plated in triplicate and left unstimulated; stimulated with a 1/30,000, 1/60,000, or 1/120,000 dilution of C305 ascites; or stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml). Cultures were incubated at 37°C for 6 h, after which time cells were harvested and lysed. The cell lysate was added to an equal volume of 2X luciferase buffer, and immediately before analysis, luciferin specific for the NF-AT reporter luciferase was added. The luminescent signal was quantitated using a TR717 Luminometer (Tropix). The reaction was stopped and samples were read a second time immediately after the addition of Renilla-specific luciferin. NF-AT luciferase values were normalized for transfection efficiency using values from the Renilla-luciferin reaction and expressed as percentage of maximal stimulation (PMA/ionomycin).

Bone marrow chimeras
SLP-76-deficient mice were housed at the University of Pennsylvania in a pathogen-specific free environment. All animal work was reviewed, approved, and performed in accordance with University guidelines. Retroviral supernatants were generated by cotransfecting 293 cells with 20 μg of MIGR1 vector and 10 μg of helper plasmid (pCL-ECO; Imgenex). Transfections were performed using 180 μg/ml polybrene (American Biosciences). Data were analyzed with FlowJo software (Tree Star). Histograms represent the average ratio of FL5/FL4 fluorescence over time.

Results
Phosphorylation of SLP-76 on Tyr145
To dissect the role of the individual N-terminal tyrosines of SLP-76 in mediating TCR signaling events, WT or tyrosine mutants of murine SLP-76 were cloned into the MIGR1 vector (27),
The MIGR1 vector allows for expression of SLP-76 and GFP using an internal ribosomal entry site. The SLP-76 tyrosines were mutated to phenylalanine individually or as combinations, as follows: Y112F, Y128F, Y145F, Y112/128F, Y112/145F, Y128/145F, and Y3F. Jurkat T cells deficient in SLP-76 expression (J14) were stably transfected with empty vector, WT SLP-76, or a SLP-76 Y→F mutant (Fig. 1A). Cells were sorted for matched GFP expression by flow cytometry. SLP-76 protein levels in reconstituted J14 cells were similar in all mutants, as measured by flow cytometry and Western blot analysis (Fig. 1, B and C).

Previous studies of Jurkat (SLP-76-sufficient) cells transiently transfected with similar tyrosine mutants demonstrated that tyrosines at positions 112 and 128 were phosphorylated following TCR stimulation, as measured by their reactivity with the phenotyrosine-specific Ab 4G10 (26). However, phosphorylation of SLP-76-containing mutations at tyrosines Y112 and Y128 showed no evidence that the tyrosine at position 145 was phosphorylated under the same assay conditions. To directly address whether Tyr<sup>145</sup> is phosphorylated in response to TCR stimulation, WT and mutant reconstituted J14 cells were left unstimulated, or stimulated with two concentrations of the anti-TCR Ab, C305, or PV. Cell mutant reconstituted J14 cells were left unstimulated, or stimulated through the TCR. Phosphorylation of the Y112/128F mutant was only detected when cells were stimulated with the phosphatase inhibitor PV, indicating that detectable TCR-induced phosphorylation of Y145 requires tyrosine residues at either position 112 or 128.

The Tec family kinase Itk has been reported to associate with SLP-76 (24, 25). Another Tec kinase, resting lymphocyte kinase (Rlk), has been shown to enhance phosphorylation of the N-terminal tyrosines of SLP-76 when overexpressed in Jurkat cells (32). To determine whether Itk or its family member Rlk is required for phosphorylation of this tyrosine residue in primary cells, purified T cells from WT, Itk<sup>−/−</sup>, Rlk<sup>−/−</sup>, or Itk<sup>−/−</sup>-Rlk<sup>−/−</sup> doubly deficient mice (33) were stimulated with plate-bound anti-CD3, and cell lysates were blotted for the presence of phosphorylated Y145. In T cells from all mice, unstimulated cells showed no Y145 phosphorylation, but phospho-Y145 was detected by 5–10 min in stimulated cells. These data indicate that neither Itk nor Rlk is required for Y145 phosphorylation (Fig. 2B). SLP-76 is a known target of the Syk family kinase, Zap70 (18, 34, 35). Using the Zap70- and Lck-deficient Jurkat derivatives, P116 (35) and JCaM1 (36), respectively, we investigated whether either of these kinases is responsible for phosphorylation of Y145. In Jurkat cells, Y145 was inducibly phosphorylated upon stimulation with PV, whereas no phosphorylation was detected in JCaM1 cells and very little phosphorylation (or none in some experiments) was detected in P116 cells (Fig. 2C). These data indicate that both of these kinases are required for optimal Y145 phosphorylation.

**FIGURE 1.** SLP-76 is expressed equivalently in J14 stable cell lines. A, Schematic of SLP-76 in MIGR1 depicts location of the functional regions of SLP-76. B, J14 stable cell lines were stained intracellularly for SLP-76 expression. A comparison of SLP-76 expression in J14 cells (shaded histogram), WT SLP-76 (black line), or tyrosine mutants (light line) is shown. C, Cell lysates from equal numbers of J14 stable transfectants were probed by Western blot for SLP-76 expression. Lanes 1–9, J14 cells transfected with empty vector (lane 1), or mutants of SLP-76, as follows: WT (lane 2), Y3F (lane 3), Y112F (lane 4), Y128F (lane 5), Y145F (lane 6), Y112/128F (lane 7), Y112/145F (lane 8), and Y128/145 (lane 9).

Of the individual tyrosines, Y145 of SLP-76 is the most critical for TCR signaling. Functionally, it has been shown that the N-terminal tyrosines of SLP-76 are important for optimal TCR-induced NF-AT activity in Jurkat cells (6, 15, 20). To determine the role of each N-terminal tyrosine for NF-AT activation, we measured the ability of the J14 stable lines to activate an NF-AT reporter. Reconstitution of J14 cells with the Y<sup>3</sup>F construct, in which Y145 is not mutated, showed minimal Ca<sup>2+</sup> flux. This diminution was greatly enhanced when paired with mutations of the Y145 residue (Y3F, Y145F, Y112/145F, and Y128/145F) did not show reactivity with the anti-Y145 phospho-specific Ab under any condition. Surprisingly, in cells reconstituted with the Y112/128F construct, in which Y145 is not mutated, phosphorylation of Y145 was not detected when the cells were stimulated through the TCR. Phosphorylation of the Y112/128F mutant was only detected when cells were stimulated with the phosphatase inhibitor PV, indicating that detectable TCR-induced phosphorylation of Y145 requires tyrosine residues at either position 112 or 128.

These results were mirrored by defects in the ability of these single and double mutants to phosphorylate PLC<sub>γ1</sub> following TCR stimulation (Fig. 4B). Of the individual tyrosines, mutation of 145 was most disruptive in the activation of PLC<sub>γ1</sub> and subsequent Ca<sup>2+</sup> flux. This diminution was greatly enhanced when paired with mutations in either Y112 or Y128, resulting in a phenotype similar to the Y3F transfectant.
The role of SLP-76 tyrosines in vivo

Taken together, these data indicate that while all three N-terminal tyrosines of SLP-76 are required for optimal SLP-76 function, mutation of Y145 has the largest impact on Ca²⁺ flux, PLCγ1, and Erk activation in vitro. To investigate the role of SLP-76 tyrosines in vivo, we generated bone marrow chimeras that expressed WT or mutant forms of SLP-76 in their hematopoietic compartments. SLP-76-deficient bone marrow or fetal liver was transduced with retroviruses generated with the MIGR1 constructs. As with transfection with MIGR1 plasmid, infection with the MIGR1-derived retrovirus results in coexpression of SLP-76 and GFP. Qualitatively similar results were found whether bone marrow or fetal liver cells were used to reconstitute irradiated Rag1−/− or B6 mice. In some experiments, WT SLP-76 was fused to GFP. Expression of fused or nonfused SLP-76 gave similar reconstitution in these bone marrow chimaera experiments (data not shown).

Mice reconstituted with SLP-76 Y145−/− bone marrow or fetal liver transduced with WT SLP-76 gave rise to mature GFP⁺ CD4⁺ T cells and GFP⁺ CD8⁺ T cells, whereas no GFP⁺ T cells were generated in mice reconstituted with bone marrow or fetal liver transduced with empty MIGR1 retrovirus. Very few T cells were generated when Y3F retrovirus was used (Fig. 6A). Because mutating the tyrosine at position 145 appeared to have the biggest impact among the single mutants in J14 cells, mice were reconstituted with the Y145F retrovirus or with the Y112/128F mutant to serve as a complement for determining the function of SLP-76 when only the Y145 is present. Both of these constructs supported CD4⁺ and CD8⁺ T cell development; however, the ratio of CD4 to CD8 in the spleen was substantially skewed toward CD8⁺ T cell development in all chimeras receiving Y145F bone marrow (Fig. 6A, graph). Skewing was also noted in the Y112/128F mice, although to a much lesser extent. Notably, we consistently found (7 of 7 mice) that the level of CD3 expression was diminished on T cells from the Y145F chimera as compared with WT levels, whereas CD3 levels were either normal or only modestly decreased (in ~50%) on either CD4⁺ or CD8⁺ cells from the Y112/128F chimera (Fig. 6B).

The functional capacity of T cells from the WT, Y145F, and Y112/128F chimeras was addressed by assessment of CD69 and CD25 up-regulation following overnight stimulation with anti-CD3 over a range of TCR doses (Fig. 6C). CD4⁺ and CD8⁺ T

FIGURE 2. Phosphorylation of Y145 of SLP-76 requires Tyr12² or Tyr12⁶, and the kinases Lck and Zap70. A, J14 stable cell lines were left unstimulated or stimulated for 4 min with a 1/1,500 or 1/5,000 dilution of C305 ascites or with PV. Western blot analysis was performed using a phospho-Y145 of SLP-76-specific Ab. Blots were stripped and reprobed for SLP-76 expression; n = 2. B, Purified T cells from WT, Itk⁻/⁻, Rlk⁻/⁻, and Itk⁻/⁻Rlk⁻/⁻ mice were stimulated on plates coated with 5 μg/ml anti-CD3 (2C11). Cells were lysed after the indicated time points, and lysates were analyzed by Western blot as in A; n = 3. C, Cell lysates from unstimulated or PV-stimulated Jurkat, P116, and JCaM1 cells were blotted for phosphorylation of Tyr145. Blots were stripped and reprobed with a phospho-Y145 of SLP-76-specific Ab. Blots were stripped and reprobed for actin as a loading control; n = 6.

FIGURE 3. The N-terminal tyrosines of SLP-76 are required for NF-AT activation. NF-AT activity was measured in the J14 stable cell lines using an NF-AT-luciferase reporter. Transfected cells were left unstimulated or stimulated for 8 h with C305 ascites (1/30,000, 1/60,000, or 1/120,000 dilutions) or with PMA and ionomycin. Luminescence from the NF-AT reporter was normalized for transfection efficiency and expressed as the percentage of maximal stimulation; n = 4.
cells from both mutant reconstituted mice had a diminished capacity to up-regulate CD69 and CD25 following TCR engagement as compared with the WT chimeras. Although the ability of the Y145F and Y112/128F mutants to support the up-regulation of CD69 in J14 was very similar (Fig. 5B), in vivo, Y145 was found to be more important for both CD69 and CD25 up-regulation especially at increasing Ag concentrations. This may be the result of differential TCR expression seen on the primary T cells from these mice. In the case of both Y145F and Y112/128F reconstitution, the CD8\(^+\)/H11001 cells were more affected than the CD4\(^+\)/H11001 cells by the lack of WT SLP-76. These data demonstrate that although expression of the Y145F and Y112/128F mutants is sufficient for the generation of T cells, their signaling capabilities are markedly diminished, with the Y145F mutant demonstrating a more severe phenotype as compared with the Y112/128F.

**Discussion**

The generation of T cells and their subsequent activation require a well-orchestrated assembly of numerous effector proteins and their proper activation. SLP-76 is an adaptor that has been shown to be central for both of these processes. In this study, we investigated the function of the N-terminal tyrosines of SLP-76 for their ability to support signals from the TCR in vitro and to allow for the development of functional T cells in vivo. We previously demonstrated that Y145 was critical for optimal NF-AT activation (26). These data were from transient overexpression of SLP-76 mutants.

**FIGURE 4.** Optimal Ca\(^{2+}\) mobilization and PLC\(_{\gamma}1\) phosphorylation require the N-terminal tyrosines of SLP-76. A, J14 stable lines were loaded with Indo-1. Baseline Ca\(^{2+}\) level was collected for 30 s, after which time C305 (1/30,000 dilution) was added. Ca\(^{2+}\) was measured for 7 min. Histograms display the ratio of Ca\(^{2+}\)-bound Indo-1 to unbound Indo-1 over time; n = 5. B, J14 stable lines were left unstimulated (lanes 1) or stimulated for 2 min (lanes 2), 10 min (lanes 3), or 30 min (lanes 4) with 1/5,000 dilution of C305. Cell lysates were probed for phospho-PLC\(_{\gamma}1\) and total Erk as a loading control; n = 6.

**FIGURE 5.** The SLP-76 tyrosines are required for full Ras/MAPK activation. A, Cells were left unstimulated (lanes 1) or stimulated with C305 ascites (1/30,000 dilution) for 2 min (lanes 2), 15 min (lanes 3), or 30 min (lanes 4). Western blots for phospho-Erk and PLC\(_{\gamma}1\), as a loading control, were performed on cell lysates; n = 3. The fold increase in phosphorylation as compared with unstimulated cells is shown. B, Cell lines were cultured with soluble C305. After overnight stimulation, cells were harvested and stained with anti-CD69. Histograms show CD69 expression on unstimulated cells (shaded area) and stimulated cells (black line).
in Jurkat cells, which express endogenous SLP-76. In this study, we used J14 cells that lack endogenous SLP-76 and showed that mutation of Y145 had the greatest impact on the activation of not only NF-AT, but also on PLC/γ1 and Ca²⁺ flux, and up-regulation of CD69 following TCR ligation.

Moreover, combining a Y145F substitution with a mutation in either Y112 or Y128 significantly augmented these deficiencies.

Before this report, evidence that Tyr145 becomes phosphorylated following TCR stimulation was lacking. In fact, we previously reported that immunoprecipitation of SLP-76 followed by blotting...
with the phosphotyrosine-specific Ab 4G10 failed to show phosphorylation of SLP-76 in a Y112/128F mutant overexpressed in Jurkat cells (26). In this study, using a more specific Y145 phospho-specific Ab, we demonstrate that Y145 is phosphorylated following TCR ligation. SLP-76 has been shown to be a substrate of Zap70 (18, 34, 35). The majority of these studies relied on 4G10 blotting to assess SLP-76 phosphorylation. To specifically ask whether Y145 was phosphorylated in a similar manner, phospho-Y145 was assessed in cell lines deficient in Lck or Zap70 expression. These data revealed that Y145 requires both kinases for optimal phosphorylation, and are consistent with Y145 being a substrate of Lck-dependent Zap70 activity. In cell lines, SLP-76 has also been shown to be a substrate of the Tec kinase Rlk (32). However, based on the ability of Itk−/−, Rlk−/−, and Itk−/− Rlk−/− T cells to phosphorylate Y145 in a similar manner as WT T cells, it does not appear that this tyrosine is a major target of the Tec family kinases.

In our analysis of the entire panel of tyrosine mutants using the phospho-Y145-specific Ab, we noticed that Y112/128F J14 cells stimulated with anti-TCR failed to reveal phosphorylation of Y145, similar to what had been previously seen with the use of 4G10. However, using a strong stimulus such as the phosphatase inhibitor PV (30), phosphorylation of Y145 was detected in this cell line. These data suggest that phosphorylation of Y112 and Y128 facilitates the phosphorylation of Y145. The mechanism for this observation is unknown; perhaps phosphorylation of these two sites relieves steric hindrance, allowing Y145 to be more easily phosphorylated. Alternatively, perhaps binding of proteins to Y112 or Y128 stabilizes SLP-76 at the proper location, thereby allowing for efficient phosphorylation of Y145.

This pattern of phosphorylation also raised questions concerning the biological effects that mutation of these tyrosines had on TCR signaling. In several assays performed in J14 cells and in primary T cells, the Y145F mutant had a phenotype similar to the Y112/128F mutant. This finding was somewhat perplexing given the fact that phosphorylation of Y145 was undetectable with TCR stimulation when Tyr112 and Tyr128 were altered to phenylalanine. Given this information, we predicted that the phenotype of the Y112/128F mutant would be as severe as the Y3F mutant in which all tyrosines, 112, 128, and 145, were mutated to phenylalanine. These data present several interesting possibilities. The first is that Y145 is still phosphorylated in response to TCR stimuli in cells expressing the Y112/128F mutation, but that this phosphorylation is below the level of detection with our current reagents. Perhaps even low levels of Y145 phosphorylation allow for sufficient recruitment of associated molecules to provide some, albeit reduced, function. Another intriguing possibility is that Y145 is a critical residue for SLP-76, but that it functions in both a phosphorylation-dependent and -independent fashion. Thus, even when Y112 and Y128 are altered and Y145 is less amenable to phosphorylation, Y145 may still serve an important function. This may relate to binding of proteins to SLP-76 in a tyrosine- but nonphosphotyrosine-dependent manner or may relate to effects of the presence of phenylalanine on other functions of SLP-76.

These points beg the question as to what is the primary protein binder(s) for position Y145 and how these associations are affected by loss of tyrosines Y112 and Y128. SLP-76 was shown to interact with the SH2 domain of Itk in primary T cells, pointing to the N-terminal tyrosines of SLP-76 as the potential binding sites for Itk. Shortly thereafter, Bunnell et al. (24) demonstrated that this interaction could be interrupted with phosphopeptides matching the tyrosine motifs surrounding Tyr145 or Tyr112. In these experiments, only the highest doses of peptides blocked the SLP-76/Itk interaction, and the phospho-Y145 peptide was only minimally more effective than the phospho-Y112 peptide. In J14 cells expressing WT SLP-76, we also find that SLP-76 can associate with Itk when cells are stimulated with PV, and often a basal interaction was detected (data not shown). However, when we attempted to map the binding site to particular tyrosines of SLP-76, we did not see a striking or reproducible diminution of SLP-76/Itk association in the Y3F, Y145F, or Y112/128F mutant cell lines. We are continuing to pursue this important question and hope to address it through the use of primary T cells from mice expressing specific tyrosine mutants. We speculate that there may be additional proteins that associate with Y145 either in a phosphorylation-dependent or phosphorylation-independent manner. It should be noted that SLP-76 and Itk have also been shown to interact through the Itk-SH3 domain and a defined sequence in the proline-rich region of SLP-76 (24). Thus, although the requirements of Y145 for Itk binding may be somewhat unclear, phosphorylation of SLP-76 at the N-terminal tyrosines and assembly of a macromolecular complex, which includes Itk, are critical for function.

All three tyrosines in the N terminus of SLP-76 contribute to the activation of each pathway tested in this study, although the degrees to which each of these pathways are affected vary among the mutants. These data suggest that the N-terminal tyrosines of SLP-76 support overlapping signals downstream of the TCR. In this role, SLP-76 anchors Vav/Nck at positions Y112 and Y128 and Itk through other SH2- and SH3-dependent interactions. Independently, these effector molecules can lead to the partial activation of TCR signaling events such as PLCγ1 phosphorylation. However, for full activation, phosphorylation of all three tyrosines and presumably recruitment of all SLP-76 binders are required. Conversely, although all of the pathways investigated in this study were affected by each of the N-terminal tyrosines of SLP-76, it is possible that distinct signaling pathways, which were not assessed in this study, are mediated by these individual tyrosines. In fact, in mast cells, degranulation was shown to be highly dependent on Tyr145, whereas mutating Y112/128F had no effect. Conversely, the pair Y112/128 was absolutely necessary for JNK phosphorylation, but Y145 was dispensable (38).

When Tyr145 was mutated in vivo, both CD4SP and CD8SP T cells could still be generated. However, the ratio of CD4SP to CD8SP cells was significantly decreased due to an increase in the percentage of CD8SP cells. The nature of this increase in CD8SP cells is unknown. It is possible that these cells are undergoing expansion in the periphery. If this is the case, it is via a mechanism that does not lead to up-regulation of CD69 or CD25, or an increase in cell size. Alternatively, the CD4SP to CD8SP ratio may be established in the thymus. The small size of the thymus in these chimeras (including the WT SLP-76 and Y112/128F chimeras) limited analysis of this organ; however, preliminary data suggest that this may be the case. This possibility is intriguing given that weaker (39–41) and/or shorter signals (42, 43) during thymic development appear to favor CD8 T cell development. Indeed, based on data from the J14 studies, we would predict that weaker signals would be generated in thymocytes expressing the Y145F mutation. Again, establishment of mice in which these mutations are knocked in will allow for a more extensive examination of how thymocyte development is affected by alterations of these tyrosines.

Tyr112 and Tyr128 of SLP-76 are responsible for binding Vav, and appreciable binding is thought to require both residues (20). Jurkat cells that lack Vav1 expression show severe defects in NF-AT activation and CD69 up-regulation and have protracted Ca2+ flux following TCR ligation (44). This phenotype is similar to that described in this work for the J14 Y112/128F mutant cell line. Deletion of Vav1 in mice results in a more severe phenotype. There is a reduction in the number of T cells in these mice, and
they show minimal TCR-induced Ca\(^{2+}\) flux and proliferation (45, 46). Based on these data, loss of tyrosine phosphorylation on Y112 and Y128 results in a phenotype similar to, but not as severe as that seen in T cells deficient for Vav. This suggests that Vav is involved in other T cell pathways that are independent of SLP-76, or that Vav can mediate some of its functions without directly interacting with SLP-76.

Given the similarity of signaling capabilities between the Y145F and Y112/128F J14 mutants (with the Y112/128F having a stronger defect in some assays), a logical prediction would have been that T cells from the Y112/128F chimera would be less capable of activation than the Y145F T cells. One explanation may be due to the lower levels of CD3 expressed on T cells from the Y145F chimeras as compared with Y112/128F chimeras. This parameter was not a factor in the J14 studies inasmuch as these cell lines were monitored for uniform TCR expression. Alternatively, there may be signals required during development that are downstream of Y114; these signals may impact the subsequent ability of peripheral T cells to function, and may not be absent with the loss of Y112/128.

The data presented in this study reinforce the importance of the N-terminal tyrosines of SLP-76 in T cell function and development. The involvement of these residues in similar pathways emphasizes the role that SLP-76 has in coordinating the assembly of molecules for the generation of a full TCR signal. It also points to SLP-76 as a rheostat that could be manipulated to modulate the strength of signal perceived by the T cell. How these differences in signal transduction (as measured in a cell line) are interpreted by the developing T cell was revealed by in vivo reconstitution of SLP-76-deficient mice. Although chimeras generated with retroviral reconstitution of SLP-76 provide valuable insight into the ability of these SLP-76 tyrosine mutants to support in vivo T cell development, variability in the degree of chimerism established in each mouse limited the scope of in vivo analysis. Thus, to understand the mechanisms underlying the different phenotypes observed in the Y145F vs Y112/128F chimeras, a genetically stable knock-in model will be required. Such a system would allow for both thymic and peripheral T cell populations that develop under normal developmental conditions to be analyzed on both a cellular and molecular level.

Acknowledgments

We thank Natalie Bezman, Farhad Abtahan, and Marielena Vélez for help with bone marrow chimera studies; Avnish Bhandoola for helpful discussions; and Benjamin Olsenchock and Drs. Jonathan Maltzman and Gaele Mesnace for critical reading of this manuscript.

Disclosures

The authors have no financial conflict of interest.

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