

Differential Requirement for SLP-76 Domains in T Cell Development and Function

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Summary

The hematopoietic cell-specific adaptor protein, SLP-76, is critical for T cell development and mature T cell receptor (TCR) signaling; however, the structural requirements of SLP-76 for mediating thymopoiesis and mature T cell function remain largely unknown. In this study, transgenic mice were generated to examine the requirements for specific domains of SLP-76 in thymocytes and peripheral T cells *in vivo*. Examination of mice expressing various mutants of SLP-76 on the null background demonstrates a differential requirement for specific domains of SLP-76 in thymocytes and T cells and provides new insight into the molecular mechanisms underlying SLP-76 function.

Introduction

Understanding how the pre-TCR and $\alpha\beta$ TCR direct maturation, selection, and activation events in thymocytes and T cells has been an area of intense investigation. Although these processes require common molecular pathways, precisely how these signals are integrated and translated into distinct programs is not completely understood. Adaptor proteins, which lack intrinsic enzymatic activity, play an integral role in organizing molecular events into productive signals required for the development and function of diverse cell types. SH2 domain-containing leukocyte-specific phosphoprotein of 76 kDa

(SLP-76) is an adaptor protein essential for thymocyte development (Clements et al., 1998b; Pivniouk et al., 1998), signaling through the mature TCR (Yablonski et al., 1998), collagen-induced platelet activation (Clements et al., 1999), and Fc ϵ R-dependent mast cell activation (Pivniouk et al., 1999).

SLP-76 consists of three protein interacting domains (Figure 1A). Its amino terminus carries tyrosine residues, which become phosphorylated by syk-family protein tyrosine kinases (PTKs) following TCR engagement (Wardenburg et al., 1996). The central proline-rich region mediates an interaction with the SH3 domain of the Grb2 homolog, Gads (also known as Grf40, GrpL, Mona, or GRID) (Asada et al., 1999; Bourette et al., 1998; Ellis et al., 2000; Law et al., 1999; Liu et al., 1999). The carboxy-terminal SH2 domain associates with another adaptor protein, ADAP (previously known as SLAP-130/Fyb), upon TCR-dependent ADAP phosphorylation (da Silva et al., 1997; Musci et al., 1997a). Additionally, an interaction between the SLP-76 SH2 domain and tyrosine-phosphorylated HPK1 has been recently characterized (Sauer et al., 2001). Following TCR engagement, the ZAP-70 PTK becomes activated, resulting in phosphorylation of several substrates, including SLP-76 and the raft-associated transmembrane adaptor protein Linker for Activated T cells (LAT) (Zhang et al., 1998). Tyrosine phosphorylation of SLP-76 enables its interactions with the SH2 domains of the Rac/Rho guanine nucleotide exchange factor, Vav (Fang and Koretzky, 1999; Raab et al., 1997); the adaptor protein, Nck (Bubeck Wardenburg et al., 1998); and the Tec-family PTK, Itk (Bunnell et al., 2000; Su et al., 1999). In addition, the SH2 domain of Gads associates with phosphorylated LAT (Asada et al., 1999; Law et al., 1999; Liu et al., 1999), thereby recruiting SLP-76 and its associated molecules into lipid rafts.

Mutant Jurkat T cell lines deficient in either SLP-76 or LAT exhibit defects in TCR-induced ERK activation, PLC γ 1 phosphorylation, and calcium influx, resulting in an inability to activate IL-2 NFAT/AP-1 promoter activity (Finco et al., 1998; Yablonski et al., 1998; Zhang et al., 1999a). In addition, mice deficient in SLP-76 or LAT demonstrate a complete block in thymocyte development at the CD4⁺CD8⁻DN stage (Clements et al., 1998b; Pivniouk et al., 1998; Zhang et al., 1999b), likely resulting from defective signaling downstream of the pre-TCR. Furthermore, a similar role for SLP-76 and LAT in transducing signals downstream of syk-family PTKs has also been established in other hematopoietic cell types, including platelets (Asazuma et al., 2000; Clements et al., 1999; Gross et al., 1999; Pasquet et al., 1999) and mast cells (Pivniouk et al., 1999; Saitoh et al., 2000). From these and other data, it is suggested that SLP-76 and LAT associate to organize a multimolecular complex capable of modulating upstream TCR-induced signaling events. Although studies have demonstrated an essential role for SLP-76 in mediating T cell development and TCR signaling, the structural requirements of SLP-76 for regulating these events is not well understood. In Jurkat T cells, overexpression of SLP-76 mutants in which one

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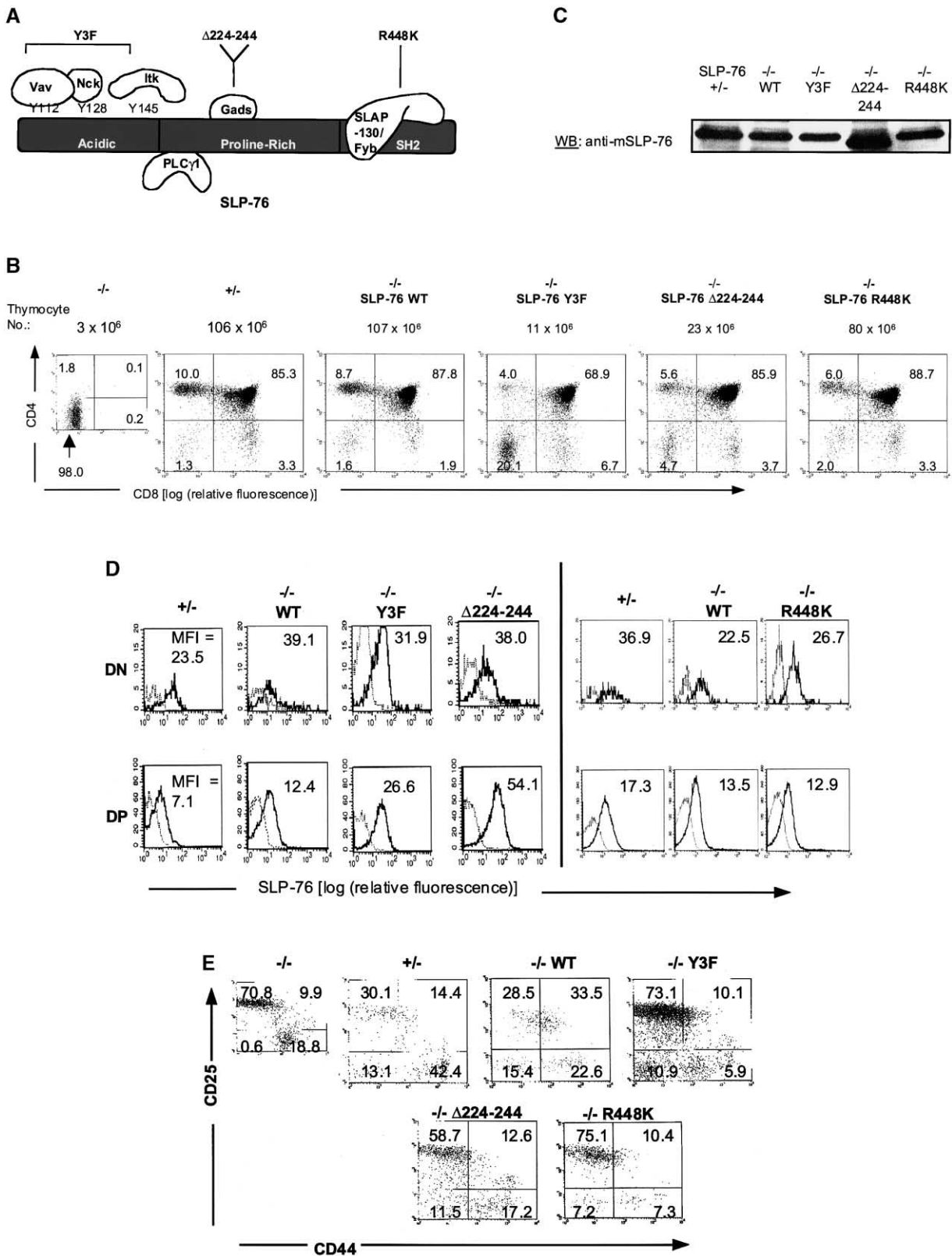


Figure 1. Effects of SLP-76 Mutations on Thymocyte Development

(A) Schematic of SLP-76 and its associated proteins.

(B) Thymocytes obtained from 8- to 22-week-old SLP-76^{-/-} (inset), SLP-76^{+/-}, and SLP-76^{-/-} mice reconstituted with WT, Y3F, Δ224-244, or R448K SLP-76 transgenes were stained with anti-CD4 and anti-CD8. Percentages of positive cells within each quadrant are indicated. Average thymocyte numbers for the various samples are indicated.

of its three structural domains has been mutated results in an inability to augment TCR-induced IL-2 promoter activity, suggesting a required role for each domain in mediating SLP-76 function in TCR-dependent signals (Fang et al., 1996; Musci et al., 1997b). However, these studies only allowed examination of SLP-76 mutants in the presence of endogenous wild-type protein, and the mechanism underlying the function of each domain was unclear. More recently, expression of these SLP-76 mutants in SLP-76-deficient Jurkat T cells demonstrated partial reconstitution by each mutant while also revealing a newly defined role for additional sequences within the proline-rich region of SLP-76 required for binding to PLC- γ 1 (Figure 1A and Yablonski et al., 2001). Although these studies have provided important insight into the mechanism underlying SLP-76 function, there remain limitations to cell line models. Indeed, studies have established differences in the requirement for specific signaling pathways between thymocytes and mature T cells (Nishina et al., 1997; Schaeffer et al., 1999; Sun et al., 2000). Moreover, current systems have not allowed for the investigation of differential requirements for distinct domains of SLP-76 in regulating thymopoiesis and peripheral T cell function in vivo. To address these issues, we generated transgenic mice that use the CD2 promoter to express either wild-type or mutant transgenes of SLP-76 exclusively in the T lineage compartment (Festenstein et al., 1996; Greaves et al., 1989; Hansal et al., 1998; Zhumabekov et al., 1995). These mice were mated onto a SLP-76-deficient background. In this report, we demonstrate that, whereas wild-type SLP-76 rescues T cell development and T cell function on the SLP-76^{-/-} background, a mutant of SLP-76 that cannot be tyrosine phosphorylated (Y3F) fails to yield normal thymopoiesis, revealing defects both in the DN-to-DP transition and in selection. A second mutant of SLP-76 (Δ 224-244), which cannot interact with Gads, demonstrates increased activity compared to the Y3F mutant but reveals a role for this region in mediating optimal SLP-76 function in thymopoiesis and T cell function. Interestingly, mice expressing a mutation of SLP-76 in its SH2 domain exhibit nearly normal thymocyte development while displaying significant defects in mature T cell function. Collectively, these data establish that there is a differential requirement for SLP-76 domains in the regulation of T cell development and function.

Results

The Amino-Terminal Tyrosines of SLP-76 Are Essential for Thymocyte Development

To define the structural requirements for specific domains of SLP-76 in thymocyte development in vivo, transgenic mice expressing various forms of SLP-76

under the control of the CD2 promoter were generated and crossed onto the SLP-76^{-/-} background. The CD2 promoter/enhancer was used to direct transgene expression in mature T cells as well as thymocytes throughout all stages of development (Festenstein et al., 1996; Greaves et al., 1989; Hansal et al., 1998; Zhumabekov et al., 1995). SLP-76-deficient thymocytes (transgene negative) show an accumulation of cells at the DN stage, concordant with the developmental arrest previously observed (Figure 1B, inset) (Clements et al., 1998b; Pivniouk et al., 1998). SLP-76^{-/-} mice expressing a wild-type SLP-76 transgene (Figure 1B), show complete reconstitution of normal T cell development as measured by CD4/CD8 surface expression and thymic cellularity (average = $94 \times 10^6 \pm 27.3 \times 10^6$, n = 7 compared to SLP-76^{+/-}: average = $93 \times 10^6 \pm 32.2 \times 10^6$, n = 10). These data demonstrate that the absence of SLP-76 expression is responsible for the arrest in thymocyte development and serve as proof of principle for subsequent SLP-76 structure-function studies.

In contrast to the complete rescue observed with wild-type SLP-76, transgenic expression of an SLP-76 mutant bearing tyrosine-to-phenylalanine substitutions at Y112, Y128, and Y145 (Y3F) yields a marked defect in thymic maturation as indicated by a significant accumulation of DN cells (15%–21% DN cells versus 1%–2% in the WT and SLP-76^{+/-} controls). In addition, total thymocyte number is significantly reduced compared to control mice (average = $10.1 \times 10^6 \pm 5.0 \times 10^6$, n = 8). Those thymocytes that progress to the single-positive (SP) stage are skewed toward the CD8 lineage. Mice expressing the SLP-76 Δ 224-244 mutant also exhibit significant abnormalities in both thymic cellularity (average = 27.2×10^6 thymocytes $\pm 13.2 \times 10^6$, n = 8) and in maturation (4%–5% DN cells), yet to a lesser degree than that observed in Y3F-expressing mice. Although the tyrosine residues and the Gads binding domain of SLP-76 appear to play critical roles in thymopoiesis, expression of the SLP-76 R448K mutant is sufficient to reconstitute nearly normal thymic cellularity (average = $83 \times 10^6 \pm 3 \times 10^6$, n = 4) and CD4/CD8 surface profiles similar to those observed in SLP-76^{+/-} littermate or transgenic SLP-76 WT-expressing mice, indicating that thymopoiesis can occur independently of the SLP-76 SH2 domain. Failure to reconstitute thymocyte development in Y3F- and Δ 224-244-expressing thymocytes is not the consequence of insufficient expression of the transgenes. Similar expression levels of each transgene are shown by Western blot analysis of total thymocyte lysates (Figure 1C) and by intracellular staining for SLP-76 protein within specific thymocyte populations (Figure 1D).

Markers for thymocyte development were characterized further by examination of the DN populations for levels of CD44 and CD25 (reviewed in von Boehmer et

(C) Lysates from 2×10^6 thymocytes were immunoblotted for SLP-76. The Δ 224-244 mutant migrates slightly faster by SDS-PAGE.

(D) CD4/CD8 double-negative (DN) and CD4/CD8 double-positive (DP) cells were analyzed for levels of intracellular SLP-76 by a combination of surface and intracellular staining. Isotype control antibody staining (dotted line); anti-murine SLP-76 staining (solid line). Mean fluorescence intensities (MFIs) of SLP-76 expression are indicated. Intracellular SLP-76 staining shown for ^{-/-} R448K thymocytes and in SLP-76^{+/-} littermate control thymocytes was done in a separate experiment.

(E) CD4/CD8/CD3 triple-negative thymocytes from the indicated mice were analyzed for surface expression of CD44 and CD25 by flow cytometry.

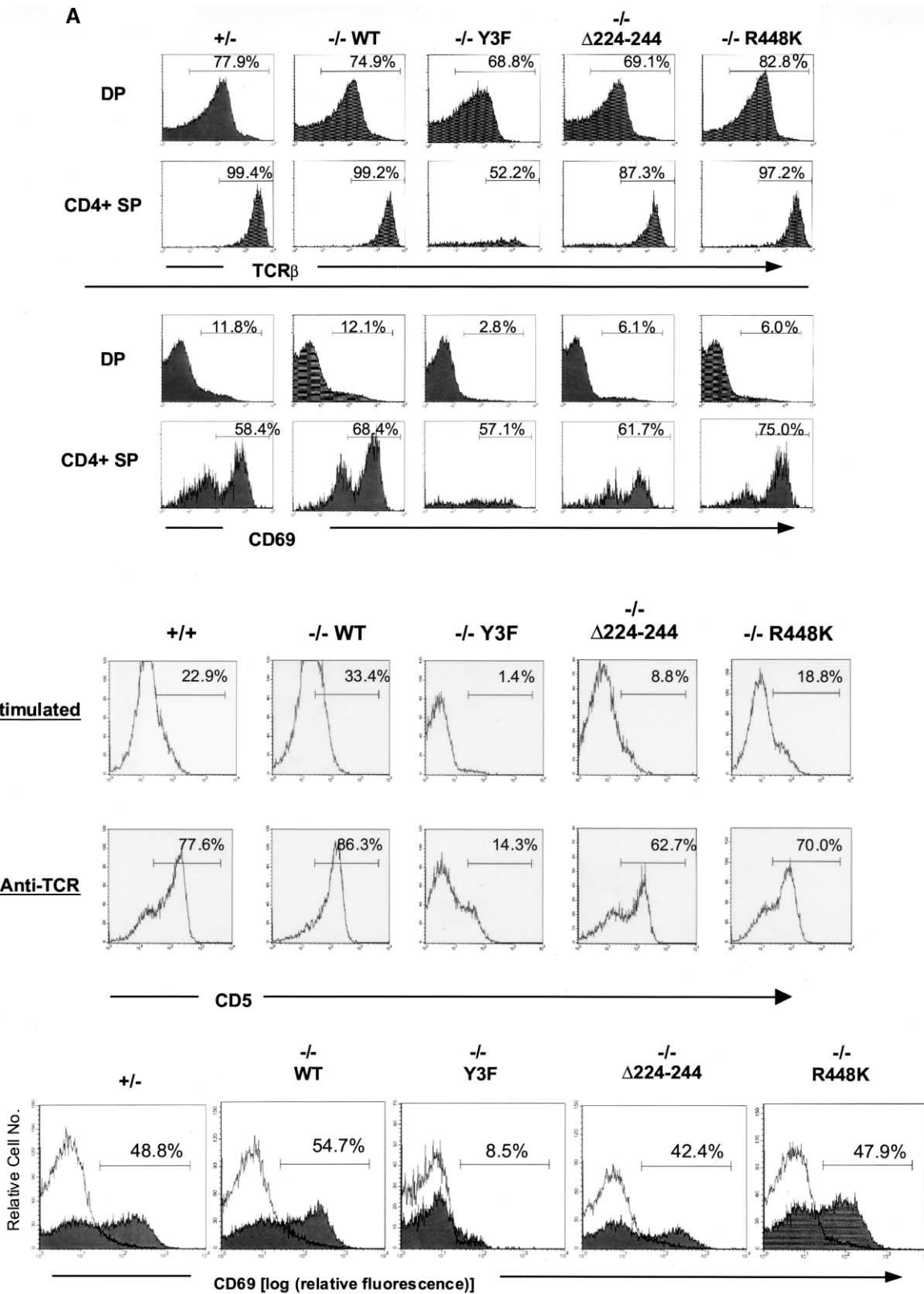


Figure 2. Defective Upregulation of TCRβ and CD69 in Y3F-Expressing Thymocytes

(A) Thymocytes from SLP-76^{+/-} mice and SLP-76^{-/-} mice expressing the indicated transgenes were stained with anti-CD4 and anti-CD8. CD4/CD8 double-positive (DP) or CD4⁺ single-positive (SP) thymocytes were analyzed for both TCRβ (top panel) and CD69 (bottom panel) expression. Percentages of positive staining cells are indicated.

al., 1999). SLP-76-deficient mice demonstrate a complete block in development at the pro-T3 (CD44⁺CD25⁺) stage (Figure 1E) (Clements et al., 1998b; Pivniouk et al., 1998), a period when pre-TCR signaling directs expansion of the developing cells and their transition to the CD44⁺CD25⁻ stage. As shown, expression of the SLP-76 WT transgene restores normal progression of thymocyte development. In contrast, both the SLP-76 Y3F- and SLP-76 Δ 224-244-expressing mutants demonstrate an accumulation of cells at the pro-T3 stage, suggesting that pre-TCR signaling events at this checkpoint are not overcome completely. Expression of the SLP-76 R448K mutant also fails to relieve the block at the pro-T3 stage entirely, indicating that the R448K mutant does not function as well as WT SLP-76 in mediating pre-TCR signals. However, in contrast to the Y3F- and Δ 224-244-expressing mutants, the SLP-76 R448K transgene is still effective at directing efficient differentiation and expansion of DP thymocytes.

Structural Features of SLP-76 Required for Thymocyte Selection

As DP thymocytes undergo positive selection in response to signaling through the mature $\alpha\beta$ TCR, TCR/CD3 and CD69 expression become upregulated. As an initial means to examine thymic selection, DP and CD4⁺ SP thymocytes from wild-type and mutant transgenic mice were assessed for TCR β and CD69 surface expression (Figure 2A). In both SLP-76^{+/-} and SLP-76 WT mice, CD69 and TCR β expression is upregulated upon transition to the CD4⁺ SP stage. In contrast, SLP-76 Y3F thymocytes show decreased TCR β and CD69 expression at both the DP and CD4⁺ SP stages, suggestive of a requirement for SLP-76 tyrosine phosphorylation in signaling events at the DP stage. Thymocytes from the SLP-76 Δ 224-244 mice demonstrate a considerable increase in surface expression of both markers at the SP stage, however to decreased levels as compared to SLP-76 WT and SLP-76^{+/-} thymocytes, consistent with suboptimal signaling through the TCR in DP thymocytes. SLP-76 R448K DP and CD4⁺ SP thymocytes exhibit both CD69 and TCR β expression levels similar to those of control and SLP-76 WT thymocytes, again suggesting that the SH2 domain of SLP-76 plays a less critical role than the amino-terminal tyrosines and Gads binding domain in thymocyte selection. In all mice examined, similar findings were also observed in CD8⁺ SP thymocytes (data not shown). Signaling through the $\alpha\beta$ TCR at the DP stage was also evaluated *ex vivo* by stimulating enriched DP thymocyte populations with plate-bound anti-TCR antibodies to mimic phenotypic changes consistent with positive selection (Cibotti et al., 1997; Groves et al., 1995; Kearse et al., 1995). Following 18–20 hr of stimulation, thymocytes were stained for the markers of selection, CD5 and CD69. As expected, thymocytes from SLP-76 WT transgenic and control SLP-76^{+/-} mice exhibit substantial upregulation of both CD5 and CD69 following TCR stimulation (Figure 2B). However,

despite comparable levels of TCR β and CD3 expression by all mice at the DP stage (Figure 2A and data not shown), DP thymocytes from SLP-76 Y3F mice demonstrate a severely attenuated response to anti-TCR stimulation as revealed by both CD69 and CD5 markers, indicating significant defects in TCR-dependent signaling required for selection.

Based upon the considerable reconstitution of thymic cellularity and CD4/CD8 surface indicators of thymocyte development, it appears that the Gads binding domain of SLP-76 may not be absolutely required for thymopoiesis but rather optimizes coupling of signaling pathways to pre-TCR and $\alpha\beta$ TCR stimulation. Accordingly, DP thymocytes expressing the Δ 224-244 mutant of SLP-76 show a reduced response to TCR stimulation, although greater than that shown by Y3F-expressing thymocytes. DP thymocytes expressing the R448K mutant upregulate both CD5 and CD69 to levels nearly equivalent to those induced by control DP thymocytes. To begin to elucidate biochemical events that may account for the observed phenotype in transgene-expressing thymocytes, we next examined TCR-induced tyrosine phosphorylation events in thymocytes from control and transgenic mice. TCR stimulation of thymocytes expressing each of the SLP-76 constructs stimulated PTK activity as indicated by the inducible phosphorylation of several proteins (Figure 3A), including a \sim 38 kDa protein (solid arrow), which presumably corresponds to LAT. Interestingly, however, several bands were absent in lysates of cells from mice reconstituted with the Y3F mutant including a protein of 76 kDa, likely SLP-76, and a molecule with an apparent molecular mass of \sim 95 kDa, a location which corresponds to Vav (broken arrows). Lysates from Δ 224-244 thymocytes show weak phosphorylation of a band migrating slightly faster than 76 kDa (curved arrow), which likely represents the SLP-76 Δ 224-244 mutant. This band is reproducibly hypophosphorylated in TCR-stimulated lysates, suggesting that association of SLP-76 with Gads, and hence LAT, may be required for optimal SLP-76 phosphorylation. Vav1 and SLP-76 have been shown to functionally cooperate in TCR signaling pathways (Fang and Koretzky, 1999; Raab et al., 1997; Wu et al., 1996). In particular, tyrosine phosphorylation of SLP-76 has been implicated in regulating TCR-induced actin rearrangements through its association with Vav1 and Nck (Bubeck-Wardenburg et al., 1998). Additionally, the gross phenotype of thymocytes from Vav1^{-/-} (Fischer et al., 1998; Holsinger et al., 1998) and Vav1^{-/-}/Vav2^{-/-} (Tedford et al., 2001) mice resembles that of SLP-76^{-/-} Y3F transgenic thymocytes. Since Vav1 has been shown to play a critical role in activation-induced TCR clustering (Fischer et al., 1998; Holsinger et al., 1998), we assessed the requirement for SLP-76 domains in ligand-induced TCR clustering in thymocytes. Thymocytes from control and transgene-expressing mice were stimulated with anti-CD3 ϵ and analyzed for TCR clustering by fluorescence microscopy (Figure 3B). Samples were also quantitatively indexed for levels of

(B) Thymocytes enriched for DP cells from each mouse were cultured in either medium alone (unstimulated) or with plate-bound anti-TCR, then analyzed for the activation markers, CD5 (top panels) and CD69 (bottom panel). For CD69 results, clear histograms represent unstimulated cells, and filled histograms represent CD69 expression following anti-TCR stimulation. Percentages of positive cells are indicated.

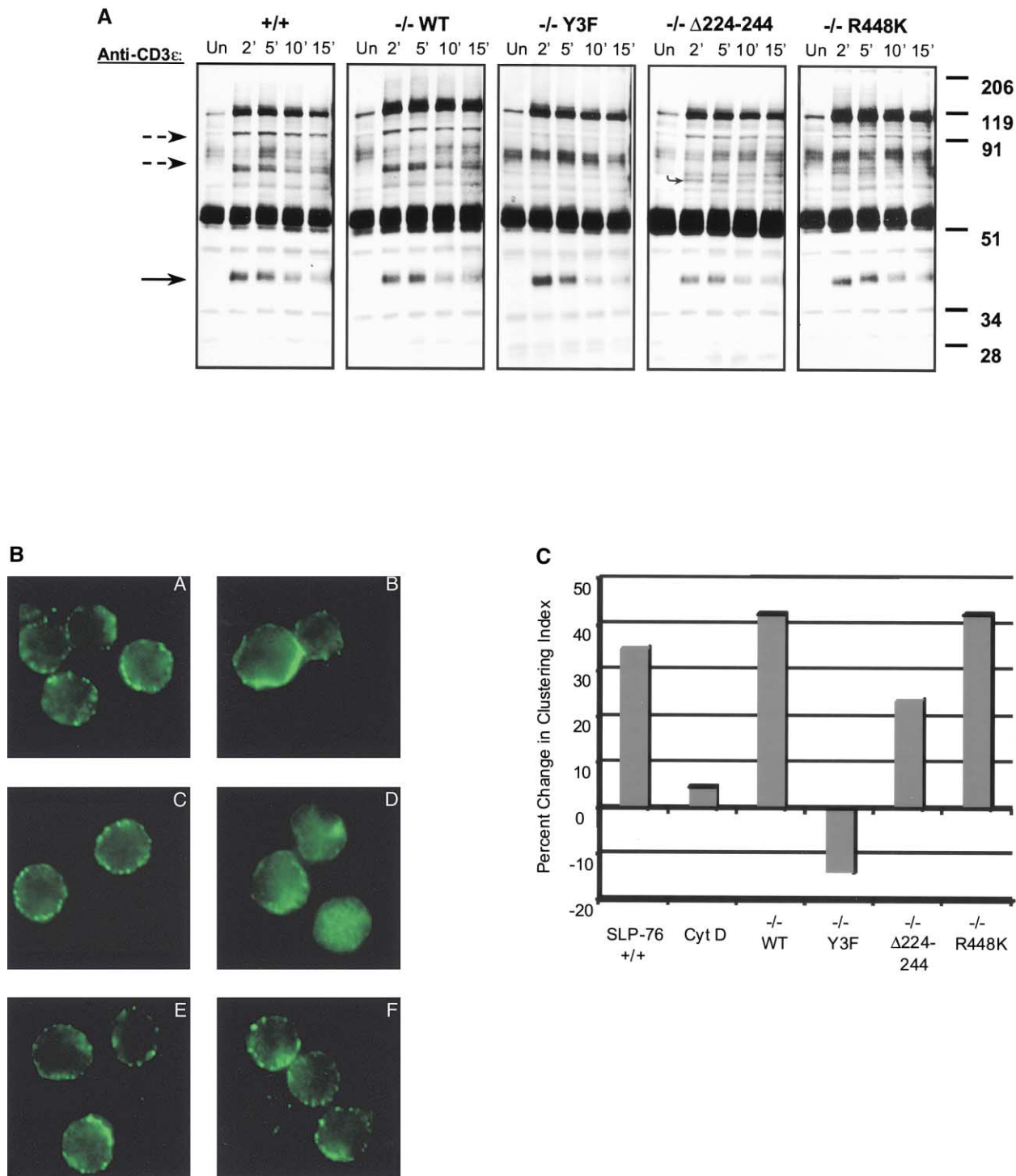


Figure 3. SLP-76 Y3F Thymocytes Signal via Their TCR but Fail to Induce CD3ε-Induced Receptor Clustering

(A) Thymocytes from SLP-76^{+/+} or SLP-76^{-/-} mice expressing the indicated transgenes were either left unstimulated (Un) or stimulated with anti-CD3ε (500A2) for the indicated times. Whole-cell lysates were resolved by SDS/PAGE, and tyrosine-phosphorylated proteins were detected with anti-phosphotyrosine monoclonal antibody (4G10). Molecular weight markers are indicated. Broken arrows indicate bands that are hypophosphorylated in Y3F-expressing thymocytes. The solid arrow at ~38 kDa indicates a phosphorylated band that is presumed to correspond to phospho-LAT. The small curved arrow in the -/- Δ224-244 panel indicates a tyrosine-phosphorylated band that likely corresponds to the mutant SLP-76, which migrates slightly faster than wild-type.

(B) Thymocytes from SLP-76^{+/+} (panel A), SLP-76^{-/-} WT (panel B), SLP-76^{-/-} Y3F (panel C), SLP-76^{-/-} Δ224-244 (panel D), and SLP-76^{-/-} R448K (panel E) mice were evaluated for TCR clustering. Panel B shows SLP-76^{+/+} thymocytes stimulated in the presence of the actin polymerization inhibitor, cytochalasin D. Representative fields from each sample in one experiment are depicted at a magnification of 1000×.

(C) Quantitative measurements of CD3ε clustering were determined by index measurements of fluorescence intensity. Results are reported as percent increase in clustering indices over that obtained for cells fixed prior to addition of crosslinking antibody. Cytochalasin D, Cyt D.

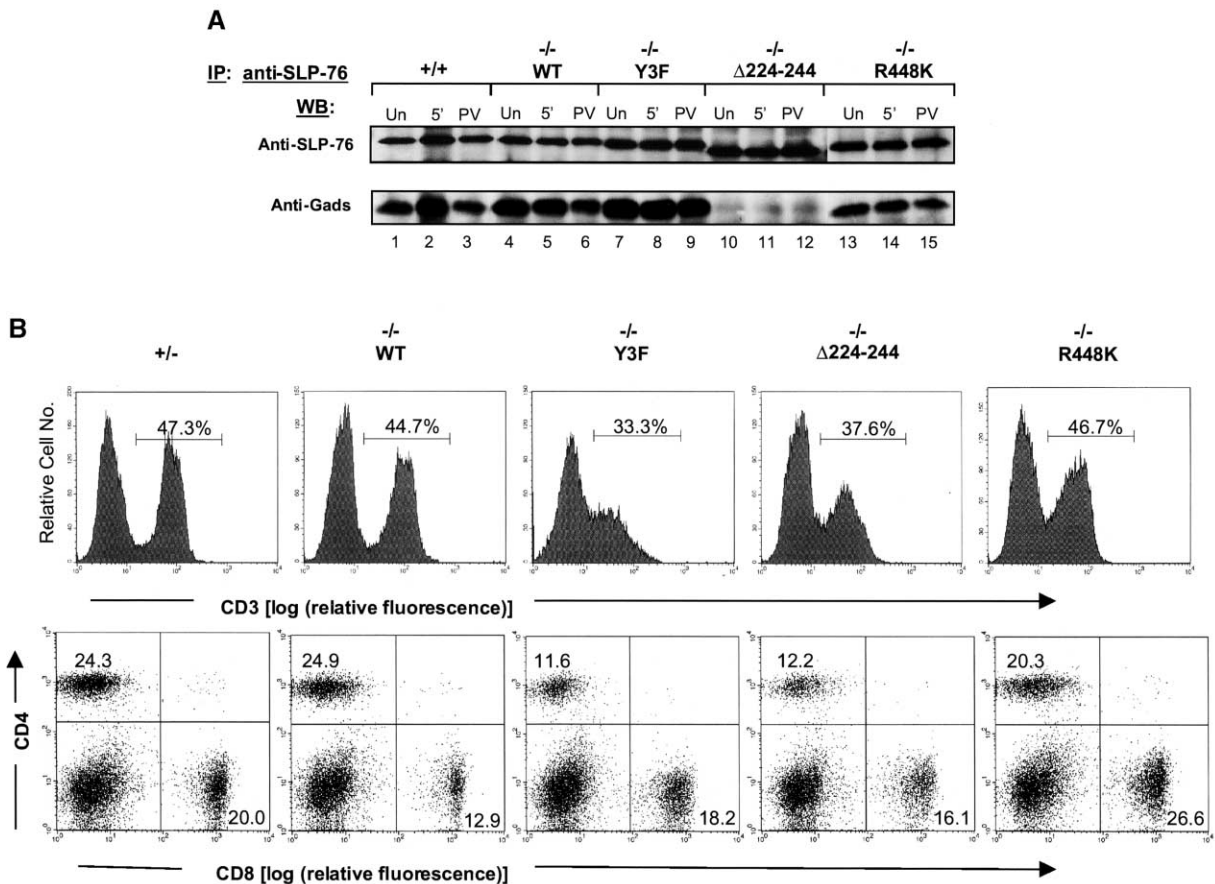


Figure 4. Effects of SLP-76 Mutations on Peripheral T Cell Populations

(A) Splenocytes from SLP-76^{+/+} or SLP-76^{-/-} mice expressing the WT, Y3F, Δ224-244, or R448K transgenes were either left unstimulated, stimulated with anti-CD3ε for 5 min, or treated with pervanadate (PV). Lysates were subjected to immunoprecipitation with anti-SLP-76 antiserum followed by immunoblot detection for Gads (bottom) or SLP-76 (top). Note that the Δ224-244 mutant migrates slightly faster than WT SLP-76 or the other SLP-76 mutants. The association between SLP-76 and Gads is constitutive and is not affected by TCR or PV stimulation.

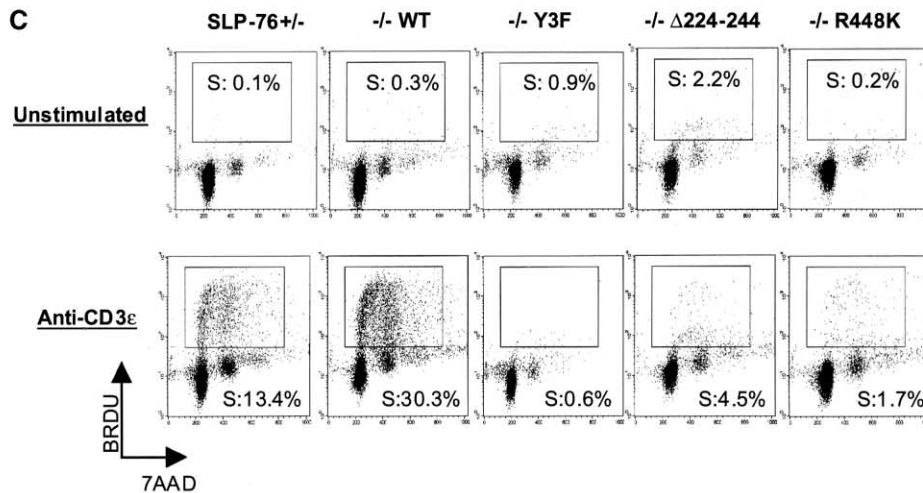
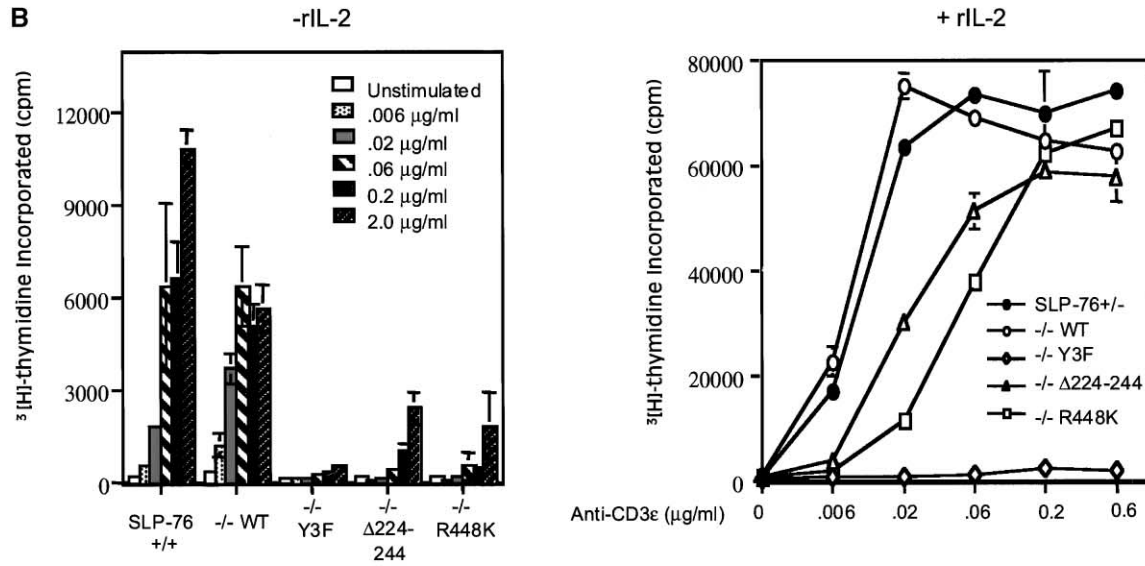
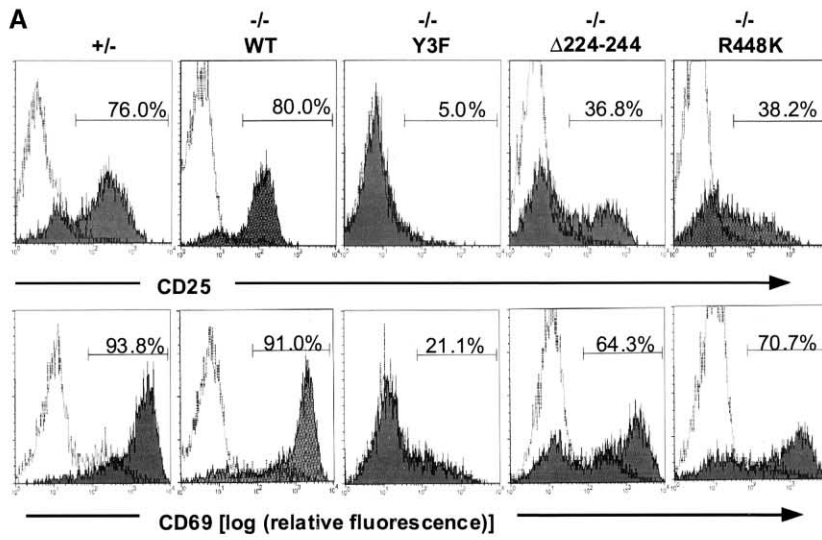
(B) Splenocytes were stained for CD3ε (histograms) or for CD4 and CD8 (bottom plots) and analyzed by flow cytometry. Percentages of positive cells are indicated.

TCR clustering by measuring the degree of deviation in fluorescence intensity normalized to the average fluorescence intensity of the cell (Figure 3C). In contrast to efficient TCR clustering exhibited by wild-type (Figure 3B, panel A) and SLP-76 WT transgenic thymocytes (panel C), Y3F-expressing thymocytes (panel D) show defects in TCR clustering similar to that seen when wild-type thymocytes are treated with cytochalasin D (panel B), an inhibitor of actin polymerization. Thymocytes from the Δ224-244 and R448K transgenic thymocytes display TCR clustering that is similar to that seen with control thymocytes (panels E and F, respectively). Collectively, these data suggest that tyrosine phosphorylation of SLP-76 plays a critical role in mediating both pre-TCR- and αβTCR-dependent selection events during thymocyte maturation. Examination of TCR-mediated receptor clustering suggests that phosphorylation of SLP-76 functions in promoting actin rearrangements during thymopoiesis, which may relate to a functional association between SLP-76 and Vav. Mutation of the Gads binding domain of SLP-76 results in less severe defects at both check-

points during thymopoiesis, implying that the association with Gads may function to regulate optimal progression throughout development. In contrast to the Y3F and Δ224-244 mutants, mutation of the SLP-76 SH2 domain permits near normal reconstitution of thymic cellularity, CD4/CD8 surface marker profiles, and expression of markers associated with thymic selection events.

Peripheral T Cell Function in SLP-76-Reconstituted Mice

Despite defects in thymocyte development exhibited by the Y3F- and Δ224-244-expressing mice, all mice revealed the presence of mature splenic T cells. To study the effects of SLP-76 domains in mature T cells, splenic T cells were isolated and examined for TCR-dependent responses. All mice express transgenic SLP-76 at levels similar to endogenous protein (Figure 4A). T cell-specific transgene expression was confirmed by intracellular staining or Western blot confirming the absence of SLP-76 protein in macrophages, platelets, and B cells (data not shown). Consistent with this, all transgenic mice main-



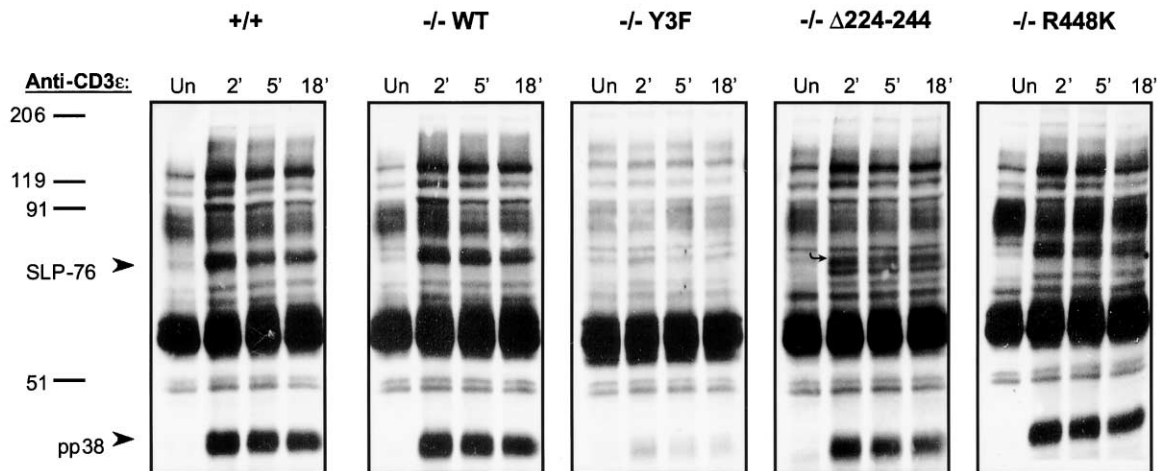


Figure 6. Y3F-Expressing T Cells Fail to Induce Protein Tyrosine Kinase Activity in Response to TCR Engagement

Purified T cells were left unstimulated or stimulated with soluble anti-CD3 ϵ (500A2) for the indicated times. Cleared whole-cell lysates were resolved by SDS/PAGE. Tyrosine-phosphorylated proteins were detected using anti-phosphotyrosine antibody (4G10). Protein molecular weight markers are indicated. The curved arrow in the Δ 224-244 panel indicates the phosphorylated band that presumably corresponds to the mutant SLP-76 protein. Arrowheads indicate observed tyrosine phosphorylated bands at \sim 76 kDa (likely SLP-76) and at \sim 38 kDa (likely LAT).

tained a similar bleeding diathesis to that observed in SLP-76 $^{-/-}$ mice as reported (Clements et al., 1999). As expected, the SLP-76 Y3F mutant protein cannot be tyrosine phosphorylated even following stimulation with the phosphatase inhibitor, pervanadate (data not shown). Additionally, whereas the Y3F and R448K mutants constitutively associate with Gads, the Δ 224-244 mutant fails to do so (Figure 4A, lanes 10–12).

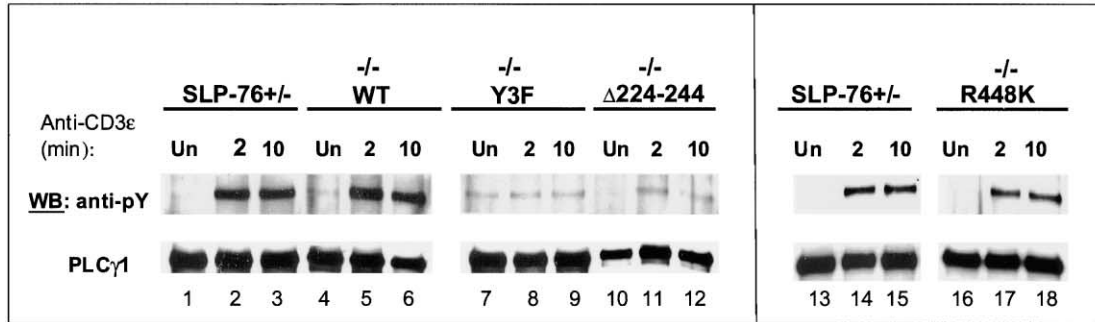
T cells from SLP-76 $^{-/-}$ Y3F transgenic mice show considerable numbers of CD3-positive T cells, although expression of the TCR is diminished (Figure 4B). More normal levels of TCR are found in mice expressing the Δ 224-244 and R448K mutants. Upon examination of CD4 $^{+}$ and CD8 $^{+}$ populations (Figure 4B), all mutant transgenic mice demonstrate a CD4:CD8 ratio that is skewed toward the CD8 lineage compared to SLP-76 WT transgenic and control mice. We find this particularly interesting in light of the fact that the CD4:CD8 skewing in the thymus is more pronounced in the Y3F-expressing mice. This disparity suggests that there exist differential requirements for SLP-76 domains in the thymus versus the periphery. Splenocytes from transgenic and SLP-76 $^{+/-}$ mice were next analyzed for TCR-induced upregulation of CD25 and CD69 following TCR stimulation. Whereas both control SLP-76 $^{+/-}$ and SLP-76 WT transgenic mice upregulate these activation antigens following treatment with anti-CD3 (Figure 5A), T cells expressing the Y3F mutant completely fail to upregulate either marker following

TCR engagement. T cells from both the Δ 224-244 and the R448K mutant mice demonstrate significantly compromised TCR-mediated upregulation of CD25 and CD69 but respond better than cells from Y3F-reconstituted mice. Treatment of splenocytes from all transgenic mice with the phorbol ester results in upregulation of both activation markers to levels similar to control SLP-76 $^{+/-}$ splenocytes (data not shown). The requirement for SLP-76 domains in mediating TCR-induced T cell proliferation was also examined. Purified T cells from SLP-76 $^{+/-}$ and transgenic mice were stimulated with varying concentrations of anti-CD3 ϵ in the presence of irradiated wild-type splenocytes. T cells from mice reconstituted with the WT transgene proliferate in response to anti-CD3 stimulation as well as control splenic T cells (Figure 5B, left panel). In contrast, splenic T cells from the Y3F transgenic mice fail to proliferate at any concentration of anti-CD3 ϵ tested. Peripheral T cells expressing the Gads binding mutant demonstrate a significantly diminished, albeit variable proliferative response to anti-CD3 stimulation, ranging from intermediate to severe defects (i.e., the fold increase in proliferation ranges between 5% and 15% of that displayed by SLP-76 $^{+/-}$ splenocytes). Surprisingly, splenic T cells from SLP-76 R448K mice also show a striking defect in proliferation at all concentrations of anti-CD3. Stimulation with anti-CD28 antibodies does not correct the T cell proliferative deficits in any of the mutant transgenic mice (data not shown);

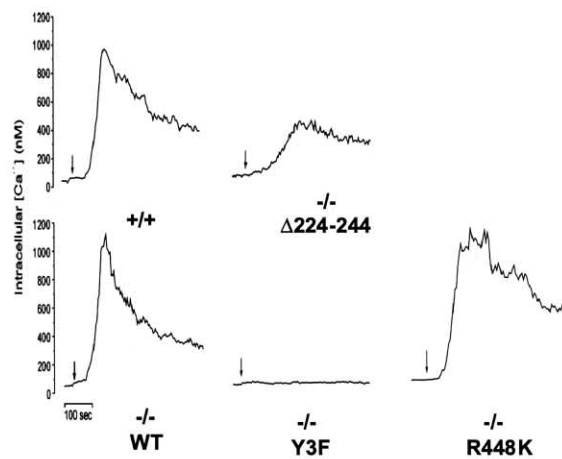
Figure 5. SLP-76 Structural Requirements for Peripheral T Cell Function

(A) Splenocytes were either left unstimulated (clear histograms) or stimulated with anti-CD3 ϵ (filled histograms) followed by analysis of CD25 and CD69 surface expression. Results are shown for Thy1.2 $^{+}$ gated cells.
(B) Purified T cells were cultured in the presence of irradiated wild-type splenocytes in either medium alone or medium containing the indicated concentrations of anti-CD3 ϵ without exogenous IL-2 (left bar graph) or with recombinant IL-2 (right panel, line graph) for 48 hr. During the last 12 hr of culture, cells were pulsed with 1 μ Ci/well of 3 [H]-thymidine prior to measurement of 3 [H]-thymidine incorporation.
(C) For BrdU experiments, splenocytes were either left unstimulated or stimulated with anti-CD3 ϵ . Cells were labeled with BrdU after 42 hr of culture followed by cell cycle analysis with 7-AAD and anti-BrdU antibody. Data represent cell cycle profiles of gated CD4- and CD8-positive T cells. Percentages of T cells in S phase are indicated.

A



B



C

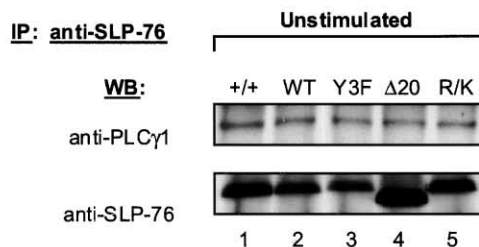
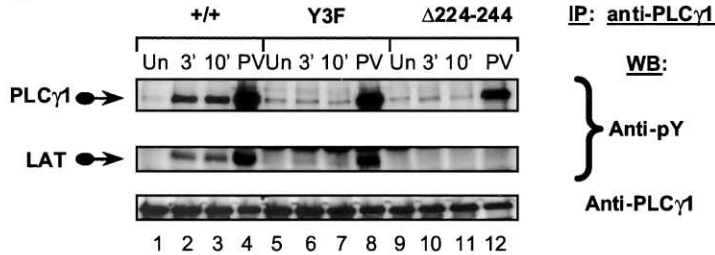


Figure 7. Effects of SLP-76 Mutations on TCR-Mediated PLCγ1 Phosphorylation and Calcium Influx

(A) Splenocytes were either left unstimulated or stimulated with anti-CD3ε. Cellular lysates were subjected to immunoprecipitation using anti-PLCγ1 followed by detection with anti-phosphotyrosine (anti-pY) antibody. Western blots were then reprobbed with anti-PLCγ1 antibody. Results from the -/- R448K and its littermate SLP-76^{+/-} control are from a separate experiment.

(B) Splenocytes from the indicated mice were stained with biotin-anti-CD3ε. Calcium signaling was initiated by addition of streptavidin (arrows).

however, T cells from all mice demonstrate a robust response to stimulation with PMA plus Ionomycin (data not shown).

Both $\Delta 224-244$ and R448K mutant transgenic T cells show a diminished ability to upregulate IL-2R α following TCR stimulation compared to control T cells (Figure 5A). Consistent with this, addition of exogenous IL-2 only partially restores their proliferative defect at lower concentrations of anti-CD3 ϵ (Figure 5B, right panel). However, at high doses of TCR stimulation, both mutant transgenic T cells proliferate similarly to control cells, suggesting that the observed defects in proliferation are not due to defective signaling through the IL-2 receptor but rather are due to defects in IL-2 production and/or IL-2R α upregulation. As expected, Y3F-expressing T cells, which fail to upregulate CD25 following TCR stimulation, also do not proliferate even with exogenous IL-2 added. To determine if the proliferative defects observed in T cells expressing mutant SLP-76 transgenes are due to a failure to enter cell cycle following TCR stimulation or an arrest in a specific phase, TCR-induced cell cycle profiles were assessed by BrdU labeling and 7-AAD analysis. In contrast to SLP-76 $^{+/-}$ and WT control T cells, mutant transgenic T cells demonstrate defective entry into the cell cycle as indicated by percentages of T cells in S phase (Figure 5C).

Proximal Signaling via the TCR in SLP-76 Transgenic T Cells

Previous studies in T cell lines implicate a requirement for SLP-76 in coupling TCR engagement to early signaling events, in particular, PLC γ 1 phosphorylation and ERK activation (Yablonski et al., 1998). To correlate the functional defects observed in the mutant transgenic with deficits in specific signaling pathways, splenic T cells were analyzed for their ability to activate various molecular events following TCR stimulation. First, purified T cells from control and transgenic mice were stimulated with anti-CD3 ϵ and assessed for tyrosine phosphorylation of cellular proteins (Figure 6). In contrast to all other transgene-expressing mice, T cells from Y3F mutant transgenic mice fail to induce detectable tyrosine phosphorylation following TCR stimulation, which is presumably due to the low levels of TCR surface expression. In contrast, both the $\Delta 224-244$ and R448K transgenic T cells induce tyrosine phosphorylation of several proteins similar to control T cells.

Tyrosine phosphorylation of PLC γ 1 results in its activation and generation of second messengers, yielding rises in free cytoplasmic calcium and PKC activation. As shown in Figure 7A (lanes 1–6), PLC γ 1 is inducibly phosphorylated in both SLP-76 $^{+/-}$ and SLP-76 WT transgenic splenocytes following CD3 engagement. In contrast, Y3F-expressing splenocytes demonstrate no

induction of PLC γ 1 phosphorylation at any time point (lanes 7–9), while T cells expressing the $\Delta 224-244$ mutant show only a poor but reproducible induction of PLC γ 1 phosphorylation (lanes 10–12). Interestingly, inactivation of the SH2 domain of SLP-76 results in induction of PLC γ 1 phosphorylation similar to wild-type control mice at both time points of stimulation (lanes 13–18).

Concordant with the PLC γ 1 phosphorylation studies, the SLP-76 WT transgenic T cells show robust TCR-induced elevations in cytoplasmic calcium (Figure 7B). T cells expressing the R448K mutation also show efficient TCR-mediated rises in intracellular calcium. As expected, T cells from Y3F transgenic mice exhibit no measurable change in cytoplasmic calcium following TCR stimulation. In agreement with the decreased PLC γ 1 phosphorylation, mutation of the Gads binding domain of SLP-76 results in a diminution in the peak elevation in cytoplasmic calcium measured.

Reconstitution studies performed in LAT-deficient Jurkat T cells suggest that recruitment of a Gads-SLP-76 complex to LAT is required for optimal association between PLC γ 1 and LAT following TCR stimulation (Lin and Weiss, 2001; Zhang et al., 2000). To determine if binding of SLP-76 to LAT is required for stable complex formation between PLC γ 1 and LAT, PLC γ 1 was immunoprecipitated from wild-type, Y3F, and $\Delta 224-244$ transgenic T cells. Following TCR or pervanadate stimulation of wild-type T cells, phosphorylated LAT can be observed in PLC γ 1 immune complexes (Figure 7C, lanes 2–4). Consistent with an inability to induce tyrosine phosphorylation events in Y3F-expressing T cells, there is an absence of PLC γ 1-associated phosphorylated LAT in TCR-stimulated lanes (lanes 6–7). However, when cells are treated with pervanadate to bypass the TCR, a band corresponding to phosphorylated LAT can be readily detected in PLC γ 1 immunoprecipitates (lane 8). Interestingly, although TCR-stimulated whole-cell lysates from $\Delta 224-244$ -expressing T cells demonstrate efficient tyrosine phosphorylation of several proteins, including LAT, the amount of LAT present in PLC γ 1 immune complexes is diminished in both TCR- and pervanadate-stimulated conditions (lanes 10–12) despite equivalent amounts of PLC γ 1 precipitated from each sample. It should be noted that in some experiments, a band corresponding to phosphorylated LAT can be detected in pervanadate-stimulated PLC γ 1 immunoprecipitates from $\Delta 224-244$ -expressing T cells; however, the amount is consistently less than that observed from wild-type or WT transgenic T cells stimulated with either anti-CD3 ϵ or pervanadate. These findings suggest that SLP-76 may be required, via its inducible binding to LAT mediated through Gads, to stabilize the interaction between PLC γ 1 and LAT. This is consistent with a constitutive interaction between SLP-76 and PLC γ 1 independent of the Gads binding domain of SLP-76, first

Curves represent averaging of signals from at least 30 individual cells.

(C) Splenocytes from the indicated mice were left unstimulated, stimulated with anti-CD3 ϵ for 3 or 10 min, or treated with pervanadate (top panel). PLC γ 1 was immunoprecipitated from lysates, and Western blots were probed with anti-phosphotyrosine antibody. The phosphorylated band of ~ 38 kDa, corresponding to LAT, is shown in the middle panel. The bottom blot is a reprobe of the stripped blot to show equal amounts of PLC γ 1 in each lane. In the bottom panel, anti-SLP-76 immunoprecipitates from lysates of unstimulated purified T cells were resolved by SDS/PAGE. The top portion of the blot was immunoblotted for PLC γ 1, while the bottom portion was probed for SLP-76. $\Delta 224-244$, $\Delta 20$; R448K, R/K.

shown in Jurkat cells (Yablonski et al., 2001) and demonstrated (Figure 7C, bottom panel) in primary T cells.

Discussion

Previous experiments in Jurkat cells showed a requirement for SLP-76 in mediating TCR-dependent signals (Yablonski et al., 1998), and *in vivo* studies also revealed a critical role for this adaptor in directing thymocyte development (Clements et al., 1998b; Pivniouk et al., 1998). Although overexpression studies have suggested a required role for each domain of SLP-76 in activating IL-2 NF-AT promoter activity (Fang et al., 1996; Musci et al., 1997b), until now *in vivo* evidence demonstrating the function of these domains has been lacking. Additionally, these previous studies precluded investigation into the role of SLP-76 domains in thymocyte development or the requirement for each domain in thymocytes versus mature T cells. By utilizing transgenic mice expressing either wild-type or mutant SLP-76, we now demonstrate a differential requirement for SLP-76 domains in mediating thymopoiesis and activation of mature T cells *in vivo*. Additionally, since the CD2 promoter/enhancer directs T lineage-specific expression, results from mice expressing the wild-type SLP-76 (SLP-76 WT) transgene confirm that the T cell defect observed in SLP-76^{-/-} mice is T cell autonomous.

We and others have shown previously that SLP-76^{-/-} thymocytes exhibit a complete developmental arrest at the CD25⁺CD44⁻ pro-T3 stage when signals from the pre-TCR are activated to promote thymocyte proliferation and TCR β allelic exclusion (Aifantis et al., 1999; Clements et al., 1998b; Pivniouk et al., 1998). To determine the importance of SLP-76 phosphorylation, tyrosine residues Y112, Y128, and Y145 were mutated to phenylalanine. Mutation of these residues results in severe defects in thymocyte development. Previous studies have shown that reconstitution of SLP-76 deficient Jurkat T cells with the Y3F mutant fails to restore normal TCR-mediated ERK activation, PLC γ 1 phosphorylation, and IP3 production (Yablonski et al., 2001). As expression of constitutively active or dominant-negative forms of members of the Ras/MAPK pathway in thymocytes indicate an important role for ERK in mediating the differentiation and expansion of DP thymocytes (Crompton et al., 1996; Iritani et al., 1999; Swat et al., 1996), we speculate that the SLP-76 Y3F mutant leads to inefficient or more transient ERK activation at the DN stage, yielding poor differentiation and expansion of DP cells. Despite this relative block, some cells still progress to the DP stage, indicating that other domains of SLP-76 are sufficient to mediate limited pre-TCR-induced signals. However, DP thymocytes expressing the Y3F mutant of SLP-76 still exhibit severe defects in selection. Of course, a comprehensive analysis of the impact of SLP-76 domains on thymocyte signaling leading to selection abnormalities will require studies utilizing TCR transgenic mice.

Following TCR ligation, SLP-76 becomes tyrosine phosphorylated by ZAP-70, resulting in its association with Vav, Nck, and Itk (Bubeck-Wardenburg et al., 1998; Bunnell et al., 2000; Fang and Koretzky, 1999; Raab et al., 1997; Su et al., 1999; Wardenburg et al., 1996). Stud-

ies performed in Jurkat cells suggest that SLP-76 is not required for Vav phosphorylation; however, we find that Y3F-expressing thymocytes fail to efficiently phosphorylate a band that migrates at ~95 kDa. Although we have not conclusively identified this hypophosphorylated band as Vav1, there are many gross similarities between Vav1-deficient mice and mice expressing the Y3F SLP-76 transgene, including a relative block in thymopoiesis at both the DN and DP stages as well as markedly decreased thymic cellularity (Fischer et al., 1998). Furthermore, similar to Vav1-deficient thymocytes, Y3F-expressing thymocytes exhibit defects in ligand-dependent TCR clustering. Thus, it is possible that SLP-76 cooperates with Vav in mediating TCR-dependent actin reorganization downstream of Vav phosphorylation. It is also possible that SLP-76 modulates other Vav-regulated functions. While Vav1 is required for TCR-dependent actin polarization leading to calcium influx, IL-2 production, and T cell proliferation (Fischer et al., 1998; Holsinger et al., 1998), it has been shown that these functions may not be entirely confined to its GEF activity for rho-family GTPases (Krawczyk et al., 2000). Interestingly, Manetz et al. have demonstrated a requirement for Vav1 in Fc ϵ R1-driven PLC γ 1/2 phosphorylation (Manetz et al., 2001). Thus, it is possible that SLP-76 and Vav1 functionally cooperate to promote TCR-mediated PLC γ 1 activation and calcium influx through effector molecules other than rho-family GTPases.

Our data demonstrate that the Gads binding region of SLP-76, although less critical than the tyrosines, is still necessary for optimal TCR and pre-TCR function. One explanation for this is that Gads mediates an inducible interaction between SLP-76 and the transmembrane adaptor, LAT (Aifantis et al., 1999; Law et al., 1999; Liu et al., 1999; Yoder et al., 2001). Significantly, the phenotype observed in Gads-deficient mice (Yoder et al., 2001), as well as transgenic mice expressing a dominant-negative mutant of Gads lacking a functional SH2 domain (Kikuchi et al., 2001), resembles the thymocyte and T cell defects observed in SLP-76 null mice expressing the Δ 224-244 mutant, supportive of a functional role underlying the SLP-76/Gads/LAT complex. It is possible that the residual signaling seen in these mice results from an association between SLP-76 and LAT independent of Gads, or alternatively that SLP-76 can be recruited to lipid rafts through yet other interactions.

Recent evidence has shown a constitutive interaction between a proline-rich region of SLP-76 and the SH3 domain of PLC γ 1 that is independent of the Gads binding domain and is critical for SLP-76 function in TCR-mediated signaling pathways (Yablonski et al., 2001). It is speculated that through this association, SLP-76 may help recruit PLC γ 1 to LAT or to stabilize the interaction, which may be required for optimal PLC γ 1 phosphorylation. In fact, our data with cells expressing the Δ 224-244 mutant of SLP-76 suggest that if SLP-76 cannot be recruited to LAT, the association between LAT and PLC γ 1 is compromised. This is true even following pervanadate stimulation, which should maximally phosphorylate both proteins. Thus, one potential function of SLP-76 may be to stabilize the recruitment of PLC γ 1 to LAT where it can then interact with lipid raft-associated substrates. This may occur via the direct interaction between SLP-76 and PLC γ 1 or via changes in conforma-

tion of either LAT or PLC γ 1, optimizing their association. We must note, however, that an alternative explanation for the phenotype of the Δ 224-244-expressing cells is that this mutant sequesters PLC γ 1 from LAT.

Surprisingly, inactivation of the SLP-76 SH2 domain reveals a differential requirement for this domain in mediating thymocyte development versus mature T cell function. SLP-76^{-/-} mice expressing the SLP-76 R448K mutant manifest nearly normal thymopoiesis as measured by cellularity, CD4/CD8 surface profiles, and upregulation of activation markers following TCR engagement. In contrast, splenic T cells from these mice demonstrate significantly compromised TCR-dependent proliferation and upregulation of CD25 and CD69 activation markers. However, SLP-76 R448K T cells exhibit normal PLC γ 1 phosphorylation, calcium signaling, and ERK activation following TCR ligation, suggesting that the SH2 domain of SLP-76 does not function upstream of these effector pathways.

The SH2 domain of SLP-76 associates with ADAP following TCR-stimulated ADAP phosphorylation (da Silva et al., 1997; Musci et al., 1997a). Recent studies of ADAP-deficient mice (Peterson et al., 2001; Griffiths et al., 2001) reveal a critical role for ADAP in TCR-dependent "inside-out" signaling to integrins. Similar to R448K-expressing T cells, ADAP^{-/-} T cells show severe defects in TCR-mediated proliferation and upregulation of activation markers while demonstrating PLC γ 1 phosphorylation, calcium influx, and ERK activation similar to wild-type T cells following TCR engagement. These data support the possibility that the phenotype observed in the SLP-76 R448K mice may be attributed, at least in part, to a loss of SLP-76 association with ADAP in TCR-dependent functions.

Finally, in addition to the three domains described in this study, it is becoming apparent that other binding motifs in SLP-76 exist and may reveal additional pathways or molecules regulated by SLP-76 in T cells and other hematopoietic cell types. SLP-76 is an adaptor protein thought to function as a scaffold, recruiting several molecules into an active nucleation complex required for initiation of pre-TCR and TCR signaling. It is becoming more evident that signaling pathways regulating thymocyte selection processes overlap with but differ from those regulating mature T cell activation. SLP-76, as well as other adaptor proteins such as LAT and Gads, may not only play an important role in regulating distinct TCR signaling pathways, but may also be critical for modulating the strength and duration of signaling, thereby regulating distinct outcomes.

Experimental Procedures

Generation of SLP-76 Transgenic Mice

Wild-type murine SLP-76 cDNA was obtained from murine splenic RNA by RT-PCR. The SLP-76 Y3F and R448K mutant cDNAs were generated by overlap extension, using primers that created either phenylalanine substitutions at amino acid residues Y112, Y128, and Y145 for the Y3F mutation or a lysine substitution at amino acid residue R448 for the R448K mutation. The SLP-76 Δ 224-244 mutant was generated by creating HindIII sites flanking amino acid residues 224-244 followed by a three-way ligation. Sequence fidelity of all cDNAs was confirmed prior to subcloning into the EcoRI and SalI sites of the CD2 promoter/enhancer provided by Dr. P. Love (National Institutes of Health, Bethesda, MD). Transgenic mice were generated by mi-

croinjection into fertilized mouse embryos derived from an F2 cross of C57BL/6J \times SJL/J (B6SJL) at the University of Iowa Transgenic Animal Facility. Animals for the transgenic SLP-76 constructs were then backcrossed onto the SLP-76 null background.

Flow Cytometry

Expression of cell surface antigens was examined by standard flow cytometric methods. Antibodies used were anti-CD3e-FITC or -PE, anti-CD69-biotin or -PE, anti-CD4-APC or -PE, anti-CD8-FITC or -PE, anti-CD25-FITC, anti-CD44-CyChrome, anti-CD5-FITC, anti-Thy1.2-APC, and anti-TCR β -CyChrome (all from Pharmingen, La Jolla, CA). Biotinylated antibodies were detected with streptavidin-APC (Pharmingen, La Jolla, CA). To detect intracellular SLP-76, cells were fixed and permeabilized using the Cytotfix/Cytoperm kit (Becton Dickinson, La Jolla, CA) according to manufacturer's directions. Cells were then stained using either a purified PE-conjugated anti-murine SLP-76 antibody (Clements et al., 1998a) or a PE-labeled goat IgG control antibody (Caltag Laboratories, Burlingame, CA). For analysis of thymocyte apoptosis, cells were stained with 7-AAD (Molecular Probes, Eugene, OR).

In Vitro Proliferation Assays

Purified T cells were cultured in 96-well plates at 4×10^4 /well with 5×10^5 irradiated splenocytes from wild-type littermate controls in triplicate with either medium alone (IMDM supplemented with 10% FBS, 10^{-5} β -mercaptoethanol, penicillin, streptomycin, and glutamine), medium containing the indicated concentrations of soluble anti-CD3e (2C11, Pharmingen, La Jolla, CA) with or without 40 units/ml exogenous rIL-2, or media containing 12.5 ng/ml PMA (Sigma, St. Louis, MO) plus 100 ng/ml ionomycin (Sigma, St. Louis, MO) for 72 hr. Cells were pulsed with 1 μ Ci/well 3 [H]-thymidine (ICN, Costa Mesa, CA) during the last 12 hr of culture. Cell cycle analysis was performed on splenocytes cultured in either medium alone or medium containing 0.1 μ g/ml anti-CD3e. Cells were pulsed with BrdU after 42 hr of culture, then analyzed for DNA content by 7-AAD staining and for incorporated BrdU with FITC-anti-BrdU.

Immunoprecipitations and Western Blots

Splenocytes or purified T cells were either left unstimulated or stimulated with 5 μ g/ml anti-CD3e (500A2, Pharmingen, La Jolla, CA) at 37°C for the indicated times or pervanadate (PV, 100 μ M NaVO₄, and 3 mM H₂O₂) at room temperature for 2 min. Lysates were resolved by SDS/PAGE for examination of all proteins or subjected to immunoprecipitation using GammaBind (Pharmacia Biotech Inc., Uppsala, Sweden) beads conjugated with either mixed monoclonal anti-PLC γ 1 antibody (Upstate Biotechnology, Lake Placid, NY) or polyclonal anti-murine SLP-76 antibody. Precipitated proteins were resolved by SDS/PAGE and transferred to nitrocellulose followed by Western blot using the indicated antibodies. Anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology, Lake Placid, NY) was used to detect tyrosine phosphorylated proteins.

In Vitro Thymocyte Stimulations

CD4⁺ CD8⁺ thymocytes were purified by two rounds of positive panning on petri plates coated with 1 μ g/ml anti-CD8 antibody (83-12-5) in PBS containing 100 mM Na₂CO₃ (pH 8.5) (Nakayama et al., 1990). For stimulations, 24-well plates were coated overnight in the presence of 0.3 ml of a 10 μ g/ml solution of purified anti-TCR β (H57-597). Cells were added at a density of 2×10^5 per well in a total of 0.5 ml culture medium (RPMI, 10% FCS, l-glutamine, nonessential amino acids, sodium pyruvate, penicillin, streptomycin, and 5×10^{-5} M 2-mercaptoethanol), then cultured for 16–20 hr prior to analysis by flow cytometry.

TCR Clustering Assays

Purified T cells were settled onto glass coverslips for 30 min followed by staining with 1 μ g/ml anti-CD3e (2C11, Pharmingen, La Jolla, CA) at 37°C in staining buffer (PBS, 1% BSA, 1% goat serum) for 30 min. Cells were then washed in PBS and stimulated with 1 μ g/ml biotinylated goat anti-hamster antibody (Jackson ImmunoResearch, West Grove, PA) for 20 min at 37°C. Stimulations were stopped by fixation in 3.7% formaldehyde. Cells were then stained with FITC-streptavidin prior to analysis using a Nikon Eclipse E800 fluorescent microscope.

As control, wild-type T cells were either stimulated in the presence of 20 μ M cytochalasin D or were fixed prior to addition of crosslinking secondary antibody. TCR clustering indices were determined by dividing the standard deviation in fluorescence intensity over the cell membrane by the average fluorescence intensity over the measured area using Metamorph Imaging software. At least 50 cells per sample were measured. Results are reported as percent increase in clustering index over that obtained for cells that were fixed prior to stimulation with crosslinking antibody.

Calcium Measurements

Single-cell calcium video imaging was performed as described (Freedman et al., 1999). In brief, cells were loaded with fura-2 AM (6.0 μ M; Molecular Probes, Eugene, OR) and concurrently labeled with biotin-conjugated anti-CD3 ϵ (2C11). Labeled cells were transferred to a temperature-controlled microscope recording chamber (Brook Industries, Lake Villa, IL) and allowed to settle onto a Poly-L-lysine (1 μ g/ml) coated glass coverslip chamber bottom. Signaling was initiated by adding streptavidin (1 μ g/ml, Jackson ImmunoResearch, West Grove, PA) to crosslink surface-bound biotin-conjugated mAb. Discrete bandwidth excitation light (340 \pm 10 nm, 380 \pm 10 nm) from a xenon source coupled to a computer-controlled monochromator (TILL, Applied Scientific Imaging, OR) was directed to the epifluorescence attachment of an inverted fluorescence microscope (Nikon TE300, USA) through a quartz fiber optic guide. Excitation light was directed through a fluorescence objective (40 \times or 100 \times , Nikon) via a dichroic mirror. The emitted fluorescence from fura-2 loaded cells passed through a 470 nm long pass filter, and images were captured with an intensified charge coupled video camera (Hamamatsu Model C2400-68) connected to the side port of the microscope using Metafluor imaging software (Universal Imaging, West Chester, PA). Fura-2 ratios were calibrated and converted to Ca²⁺ concentrations using the equation of Grynkiewicz (Grynkiewicz et al., 1985).

Measurement of T Cell Activation Markers

Splenocytes were cultured at 1×10^6 /ml in either media alone or media containing 3 μ g/ml soluble anti-CD3 ϵ (500A2; Pharmingen, La Jolla, CA) or 20 ng/ml PMA plus 0.2 μ g/ml ionomycin for 18–20 hr prior to analysis by flow cytometry as described above. Surface expression of activation markers was detected using biotinylated-anti-CD69 (APC-streptavidin) and FITC-anti-CD25. CD25 and CD69 surface expression were analyzed on gated Thy1.2-positive cells (all antibodies used in this assay are from Pharmingen, La Jolla, CA).

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