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The SLP-76 Src Homology 2 Domain Is Required for T Cell Development and Activation

Jeremy C. Burns,* Evann Corbo,‡ Janine Degen,‡ Mercy Gohil,* Christine Anterasian,* Burkart Schraven,‡§ Gary A. Koretzky,⁎⁎ Stefanie Kliche,‡ and Martha S. Jordan†

The adapter protein Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa (SLP-76) is critical for multiple aspects of T cell development and function. Through its protein-binding domains, SLP-76 serves as a platform for the assembly of multiple enzymes and adapter proteins that function together to activate second messengers required for TCR signal propagation. The N terminus of SLP-76, which contains three tyrosines that serve as docking sites for SH2 domain-containing proteins, and the central proline-rich region of SLP-76 have been well studied and are known to be important for both thymocyte selection and activation of peripheral T cells. Less is known about the function of the C-terminal SH2 domain of SLP-76. This region indubitably associates with ADAP and HPK1. Combining regulated deletion of endogenous SLP-76 with transgenic expression of a SLP-76 SH2 domain mutant, we demonstrate that the SLP-76 SH2 domain is required for peripheral T cell activation and positive selection of thymocytes, a function not previously attributed to this region. This domain is also important for T cell proliferation, IL-2 production, and phosphorylation of protein kinase D and IκB. ADAP-deficient T cells display similar, but in some cases less severe, defects despite phosphorylation of a negative regulatory site on SLP-76 by HPK1, a function that is lost in SLP-76 SH2 domain mutant T cells. The Journal of Immunology, 2011, 187: 4459–4466.

Ligation of the TCR triggers a signaling cascade that results in the activation of multiple intracellular proteins. These signals are important for proper thymocyte development upon encounter with peptide–MHC ligands present in the thymus and for the activation of mature T cells upon encounter with foreign Ags presented in peripheral organs. Propagation of TCR signals is dependent upon the formation of a multimolecular signaling complex consisting of the TCR itself, multiple effector enzymes, and adapter proteins. Adapter proteins contain no enzymatic activity but provide docking sites for other molecules critical to the function of the complex.

One adapter protein that is critical for signaling in developing thymocytes and mature T cells is Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa (SLP-76). Several domains within SLP-76 are important for its function (1). The N terminus contains three tyrosines that are necessary for the activation of Vav1, a guanine nucleotide exchange factor, Itk, a Tec family tyrosine kinase important for phospholipase Cγ1 (PLCγ1) activation, and recruitment of Nck, an adapter implicated in actin reorganization (2–7). The proline-rich region of SLP-76 mediates a constitutive interaction with the adapter Gads, which localizes SLP-76 to the plasma membrane after T cell activation (8, 9). The SH2 domain of SLP-76 serves as a docking site for a number of phosphorylated proteins including ADAP (10, 11), the serine/threonine kinase HPK1 (12), and CD6 (13), a cell surface receptor involved in T cell activation. ADAP is required for proper thymocyte selection and TCR-induced integrin activation (14, 15). In T cell lines, HPK1 has been shown to regulate positively JNK and NF-κB but to regulate negatively AP-1 and IL-2 production (12, 16–18). Recently, T cells from HPK1-deficient mice revealed a hyperactive phenotype, consistent with HPK1 acting as a negative regulator of T cell function (19).

The N terminus and proline-rich region of SLP-76 are required for TCR signal transduction. Cell lines containing mutations in these regions lead to severely diminished inositol-1,4,5-trisphosphate production, NFAT activity, PLCγ1 phosphorylation, and Ras/MAPK signaling (1, 20). In contrast to these severe defects, expression of an SH2 domain mutant of SLP-76 in cell lines results in decreased PLCγ1 phosphorylation but nearly normal inositol-1,4,5-trisphosphate production and ERK phosphorylation (20).

Determining the in vivo role for the various domains of SLP-76 has been more challenging than studies in cell lines, as SLP-76-deficient mice have a complete block in thymocyte development at the double-negative (DN3) stage (21, 22). To circumvent this limitation, SLP-76 transgenes have been used to express wild-type (WT) or mutant SLP-76 proteins specifically in T cells of SLP-76−/− mice (23, 24). In these studies, mice expressing an N-terminal or proline-rich domain mutant had thymus that were 5- to 10-fold smaller than WT thymi with greatly reduced percentages of mature single-positive (SP) peripheral T cells (24).

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Abbreviations used in this article: CBM, CARMA1–Bcl-10–MALT1; εHET, conditional heterozygous; cKO, conditional knockout SLP-76; cRK, conditional SLP-76 RK; DN, double-negative; DP, double-positive; PCC, pigeon cytochrome C; PLCγ1, phospholipase Cγ1; SH2, Src homology 2; SLP-76, Src homology 2 domain-containing protein of 76 kDa; SP, single-positive; WT, wild-type; YFP, yellow fluorescent protein.

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These cells exhibited dramatic defects in PLCγ1 activation, Ca2+ release, and proliferation (23, 24). Additional studies focused on the N-terminal tyrosines or proline-rich region of SLP-76 revealed their importance in thymocyte selection and provided a basis for the biochemical pathways that support these functions (4, 25). Unlike the gross abnormalities observed in N-terminal or proline-rich region mutants, transgenic expression of an SLP-76 SH2 domain mutant allowed for the development of mature SP thymocytes at near-normal frequencies, despite the fact that the DN3 to DN4 stage block seen in SLP-76−/−deficient mice was partially relieved (24). Moreover, peripheral T cells from these mice had normal TCR-induced Ca2+ flux, despite one report suggesting decreased PLCγ1 phosphorylation and nearly normal ERK phosphorylation (23, 24). Although proximal TCR signaling was largely preserved, T cells harboring an SH2 domain mutation failed to upregulate activation markers properly and exhibited severely defective TCR-induced proliferation (23, 24). Whereas these studies provided evidence for a role for the SLP-76 SH2 domain in peripheral T cell function, a biochemical explanation for the lack of T cell responsiveness seen in these mice has not been addressed, nor has the role for this domain in thymocyte selection been tested.

Although the transgenic approach described earlier was informative, it was hindered significantly by the fact that a large proportion of SLP-76−/−deficient mice exhibit perinatal lethality (21, 22) and by the fact that assessment of abnormalities in peripheral T cell function were potentially confounded by altered thymocyte development prior to the double-positive (DP) stage. Therefore, to investigate whether the SLP-76 SH2 domain plays a role in SP thymocyte selection and to identify how this domain supports T cell activation downstream of the TCR, we used a system that allows for regulated expression of WT or an SH2 mutant of SLP-76 during thymocyte development while circumventing the DN3 to DN4 block and perinatal lethality associated with SLP-76 deficiency. In this study, we demonstrate that the SH2 domain of SLP-76, like its binding partner ADAP, is critical for thymocyte positive selection, T cell conjugation formation, IL-2 production, and activation of the NF-κB pathway. Furthermore, we implicate PKD as a signaling intermediate downstream of both SLP-76 and ADAP. The functional defects observed in SLP-76 SH2 domain mutant T cells were more severe than those seen in ADAP−/− T cells despite the loss of phosphorylation of SLP-76 at a negative regulatory site. These data indicate that the loss of negative regulation of SLP-76 signaling imposed by HPK1 is not sufficient to overcome the loss of positive signals provided by this kinase or those supported by ADAP.

Materials and Methods

Mice

Mice that express CD4-regulated cre and have exon 3 of SLP-76 flanked by loxP sites have been described (26). Mice with mutated SLP-76 expressed under the control of a human CD2 promoter and mice deficient in ADAP have also been described (14, 24). All mice were back-crossed to C57BL/6 mice at least five times. All animal experiments were performed in accordance with University of Pennsylvania and Otto-von-Guericke University guidelines.

Flow cytometry

Thymocytes and splenocytes were harvested, washed, and stained with Abs in FACS buffer (PBS containing 2% FBS and 0.002% azide) for 30 min. Samples were collected on a FACScalibur or LSRS2 (BD Biosciences), and analysis was performed using FlowJo software (Tree Star). SLP-76 levels were determined using sheep PE-conjugated anti-mouse SLP-76. Other Abs for flow cytometry were purchased from BD Pharmingen unless otherwise noted: anti-CD25 PerCPCy5.5, anti-CD117 (c-Kit) allophycocyanin, anti-CD8 PE, Pacific blue and allophycocyanin, anti-CD4 PerCPCy5.5 and allophycocyanin-Cy5.5 (eBioscience), anti-CD69 PerCPCy5.5, anti-Vav1 PE, anti-Bcl-xL PE (Southern Biotech), and anti–TCRβ PE. PE-conjugated lineage markers were as follows: anti-CD8, anti-B220, anti-DX5, anti-NK1.1, anti–TCRγδ, anti-Mac1, and anti-Gr1.

Conjugate formation

B cells (LK35.2) were incubated at 37˚C for 1 h with or without 10 μM pigeon cytochrome C (PCC) and then washed and resuspended at a concentration of 107 cells/ml. Splenocytes were stained with anti-CD4, anti-CD8, and anti-Vav1 Abs, washed, and resuspended at a concentration of 107 cells/ml. Cells were combined at a 1:1 ratio (100 μl each) and then spun in a Beckman tabletop centrifuge for 2 min at 1200 rpm at 4˚C and incubated at 37˚C for 12 min. Conjugate formation was stopped by adding cold media containing 0.02% azide. Cells were gently mixed by pipetting three times before collection on a BD Biosciences FACSCaliber.

Cytokine, proliferation, and early activation marker analyses

T cells were purified by magnetic beads from freshly harvested splenocytes using a Dynal T cell negative isolation kit, as per the manufacturer’s protocol (114.13D; Invitrogen). Cells were stimulated overnight at 37˚C on 96-well flat-bottom plates coated with or without 10, 5, 2, or 1.25 μg/ml anti-CD3 (2C11; BD Pharmingen) and 5 μg/ml anti-CD28 (Pharmingen) for Bcl-xL, proliferation, and IL-2 assays. Supernatants were collected after 24 h and plated onto 96-well ELISA plates (Immunol 4 HBX; Thermo Scientific) coated with rat anti-mouse IL-2 (BD Biosciences) and incubated for 2 h at 37˚C. IL-2 ELISA was performed using reagents from BD OptEIA kit (555148; BD Biosciences) following the standard protocol.

Cells from overnight stimulation were harvested and stained with anti-CD4, anti-CD8, and either anti-CD69 or anti-CD25. Cells were then washed and collected on a FACSCaliber (BD Biosciences) and analyzed with FlowJo software (Tree Star). Proliferation was measured by pulsing cells after 48 h of stimulation with tritiated thymidine and harvesting after an additional 16 h.

Cell lines

SLP-76−/−deficient Jurkat (J14) cells (27) stably transfected with GFP-fused WT SLP-76, GFP-fused RK mutant SLP-76, or empty vector have been previously described (9, 28). JDAp cells were a gift from Ann Huang (Children’s Hospital of Philadelphia) (29).

Immunoprecipitations and immunoblots

T cells were purified as described earlier from freshly harvested splenocytes using magnetic bead isolation and were rested overnight at 4˚C in serum-free IMDM and then stimulated with 5 μg/ml anti-CD3 (50A2 or 2C11; BD Biosciences) and 5 μg/ml anti-CD28 (BD Biosciences). Jurkat cells were rested 1 h or overnight at 37˚C in serum-free RPMI 1640. Cells were stimulated with 1:2000 dilution of anti-TCR C305 hybridoma supernatant and 5 μg/ml anti-CD28 (BD Biosciences). Cold PBS was added to stop the stimulation. Cells were pelleted and lysed in buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris HCl (pH 7.5), 1 mM Na3VO4, 5 mM NaF, 1 mM PMSF, 5 mM Na pyrophosphate, and Protein Inhibitor Cocktail (Sigma). Immunoblots were probed with the following Abs: phospho-PKD (S744/748 and S916), phospho-IκBα (Ser32), HPK1 (all from Cell Signaling), actin (Sigma or Santa Cruz), sheep anti-mouse/human ADAP or SLP-76, SLP-76 (eBioscience) or phospho–SLP-76 (S376) (gift from V. Di Bartolo, Institute Pasteur). Blots were quantified by determining the control to target band intensity ratio followed by normalization to the intensity of target band and the 0˚ time point.

Results

Thymocyte development in SLP-76 RK mutant mice is altered

To determine the role of the SLP-76 SH2 domain in thymocyte development and function, we used mice that express an arginine to lysine mutation (RK) at aa 448, which ablates the function of this domain (30). SLP-76 RK transgenic cells were previously shown to express WT levels of SLP-76 during thymocyte development (24). Despite adequate SLP-76 expression, when mated to SLP-76−/− mice, these mice exhibit a substantial block at the DN3 stage and display increased perinatal lethality (21, 22). To circumvent these issues, we used a system that allows for conditional deletion of WT SLP-76. Conditional knockout SLP-76−/− mice expressing CD4-regulated cre-recombinase were bred to SLP-76 RK transgenic mice (26). These mice also have cre-
regulated yellow fluorescent protein (YFP) knocked into the Rosa26 locus, which enables the identification of cells expressing cre activity. The resulting conditional SLP-76 RK (cRK) mice, conditional heterozygous (cHet) mice (with one WT and one floxed SLP-76 allele), and cKO mice (with either two floxed SLP-76 alleles or one floxed and one null allele) were used for these studies. For validation of the system, the development and phenotype of cHet T cells were shown to be similar to that of WT T cells (Supplemental Figs. 1, 2).

cHet, cRK, and cKO thymocytes were analyzed for their ability to progress through the DN stages of thymocyte development. The DN profile of cRK and cKO mice is similar, with only a slightly higher percentage of DN3 versus DN4 cells observed in the mutant versus cHet mice. Thus, the DN3 to DN4 transition is substantially normalized compared with that observed in SLP-76 knockout mice (21, 22) and in SLP-76 knockout mice that express the RK transgene (24) (Fig. 1A, top row). Differentiation to the CD4+CD8+ (DP) stage occurs normally in cRK mice, as there is no difference in the percentage or absolute number of DP thymocytes in these mice compared with those in cHet mice (Fig. 1A, middle row, data not shown). However, differentiation to the CD4SP stage is significantly impaired, as measured by a decrease in the percentage and absolute number of CD4SP thymocytes in cRK mice versus cHet mice, although this defect is not as profound as that seen in cKO mice (Fig. 1A, bottom row, 1B). The defects observed in cRK differentiation are not due to decreased TCR expression (Fig. 1C) or to diminished SLP-76 expression (Fig. 1D). These data also demonstrate the efficiency of cre-mediated SLP-76 deletion, as previously reported (26), and the fidelity of the YFP reporter system.

cKO mice have fewer splenic CD4+ and CD8+ T cells compared with cHet mice and express significantly lower levels of TCR. cRK mice have normal numbers of CD4+ T cells and only slightly reduced numbers of CD8+ T cells compared with cHet mice and significantly more than that found in cKO mice (Fig. 2A, 2B). TCR levels are at or near normal on CD4+ and CD8+ T cells, respectively (Fig. 2C), and SLP-76 expression in cHet and cRK mice remains similar in peripheral T cells (Fig. 2D).

The SLP-76 SH2 domain is critical for thymocyte positive selection
To determine if the reduction in SP thymocytes was due to a defect in positive selection, we restricted the TCR repertoire by crossing conditional SLP-76 mice onto the MHC class II-restricted AND TCR transgenic background (31). The AND TCR recognizes PCC, and nearly all CD4SP T cells in these mice use the transgenic TCR. Thymi harvested from cRK and cKO AND+ mice have lower percentages and numbers of CD4SP thymocytes than their cHet counterparts, indicating defective positive selection (Fig. 3A); however, some mature CD4+ T cells expressing the transgenic TCR do develop (Fig. 3A, 3B). To gauge the function of these cells, we tested their ability to form T cell–APC conjugates. Splenocytes from mutant and control AND+ mice were incubated with PCC-pulsed B cells. Splenocytes from cRK mice form fewer conjugates than cHet splenocytes, and cKO splenocytes fail to form conjugates over background levels (Fig. 3C). Impairment of cRK T cells to form conjugates with APCs is reminiscent of a previously published report describing the phenotype of mice deficient in ADAP, an SLP-76 SH2 domain binding partner (15).

Defective T cell activation in cRK and ADAP−/− mice
Given the similarities in the defects observed in positive selection and conjugate formation between cRK and ADAP−/− mice, we directly compared the activation of purified T cells from these mice after TCR stimulation (Fig. 4A). Uptregulation of the very early activation marker CD69 is markedly reduced in cRK and absent in cKO T cells. CD69 is upregulated in ADAP−/− cells;
however, at lower titrations of anti-CD3 triggering, CD69 levels are reduced compared with those of WT controls. Upregulation of CD25 is absent in both cRK and cKO T cells, even at the highest dose of anti-CD3, whereas ADAP^2^-^2^- T cells upregulate CD25, although to a lesser extent than WT T cells.

Bcl-xL, an NF-kB target gene, was identified recently as a downstream target of ADAP after TCR stimulation (32). To determine whether the SH2 domain of SLP-76 is also required for this function, Bcl-xL upregulation in response to TCR and CD28 stimuli was analyzed. cHet and WT T cells show robust induction of Bcl-xL, whereas cRK, cKO, and ADAP^2^-^2^- T cells fail to do so (Fig. 4A). Proliferation of cRK, cKO, and ADAP^2^-^2^- T cells is also defective (Fig. 4B). cRK cells demonstrate a similar response to cKO cells in their near complete failure to proliferate, whereas ADAP^2^-^2^- cells proliferate less than the WT control but to a greater extent than cRK T cells. IL-2 production largely mirrors the proliferation data, with cRK and cKO cells producing barely detectable levels of cytokine (Fig. 4C). Similar to a previous report (14), ADAP^2^-^2^- cells also failed to produce levels of IL-2 comparable to those of WT cells (Fig. 4C). These data show that the activation phenotype of cRK and ADAP^2^-^2^- T cells is similar; however, in all cases, cRK T cells respond less well to TCR engagement than ADAP^2^-^2^- T cells.

The SLP-76 SH2 domain and ADAP are required for PKD activation

To investigate possible molecular mechanisms responsible for the defects observed in RK mutant and ADAP^2^-^2^- T cells, we analyzed signaling pathways downstream of the TCR using cell lines and primary T cells with an SLP-76 RK mutation or ADAP deficiency. For these studies, SLP-76-deficient Jurkat cells (J14 cells) stably expressing GFP alone or encoding GFP fused to WT or RK SLP-76 were generated. Cell lines express similar amounts of SLP-76 (Supplemental Fig. 3A), and mutation of the SH2 domain abrogates the inducible association of SLP-76 with ADAP and HPK1 as previously shown (10–12) (Supplemental Fig. 3B).

The serine/threonine kinase PKD has been shown to regulate TCR-induced integrin activation and to be required for IL-2 production (33, 34). Given the adhesion and IL-2 production defects present in RK mutant and ADAP-deficient T cells, we analyzed PKD activation after TCR ligation in WT and mutant T cells. Activation of PKD is associated with phosphorylation of two serine residues within its activation loop by protein kinase C family members and an autophosphorylation site within its C terminus (35, 36). PKD phosphorylation at the activation loop and autophosphorylation sites is dependent upon SLP-76, as these sites are robustly phosphorylated in WT SLP-76 reconstituted J14 cells and nearly absent in cells lacking SLP-76 (Fig. 5A, left panel). J14 cells expressing the SLP-76 SH2 domain mutant show a reduction
in PKD phosphorylation at both sites demonstrating a requirement of this domain for PKD activation (Fig. 5A, left panel). Primary T cells from cRK mice also show reduced PKD phosphorylation compared with that of cHet T cells (Fig. 5A, right panel). In all cases, decreased phosphorylation of PKD was not due to diminished PKD expression (data not shown). To determine whether T cells deficient in ADAP share this reduced PKD phosphorylation phenotype, we evaluated the PKD phosphorylation status in JDAP [an ADAP-deficient Jurkat mutant (29)] and ADAP^−/− T cells. Compared with WT Jurkat T cells, phosphorylation of PKD in JDAP cells is diminished (Fig. 5B, left panel). ADAP^−/− primary T cells also have an overall decreased magnitude of PKD phosphorylation, but prolonged phosphorylation at the autophosphorylation site is frequently seen (Fig. 5B, right panel). Although the impact of this pattern of phosphorylation on T cell activation is unclear, it is not reflective of differences in total PKD levels between WT and ADAP^−/− T cells (data not shown). These data demonstrate that normal PKD phosphorylation is dependent on the SLP-76 SH2 domain and ADAP; however, the mechanism by which these mutations regulate PKD may be different given their distinct pattern of PKD phosphorylation.

The SLP-76 SH2 domain regulates activation of the NF-κB pathway

ADAP was shown recently to regulate the activation of NF-κB by regulating the assembly of the CARMA1–Bcl-10–MALT1 (CBM) complex (37). Although it was suggested that this function of ADAP is SLP-76 independent, the shared defects between cRK and ADAP^−/− T cells in PKD phosphorylation at both sites demonstrating a requirement of this domain for PKD activation (Fig. 5A, left panel). Primary T cells from cRK mice also show reduced PKD phosphorylation compared with that of cHet T cells (Fig. 5A, right panel). In all cases, decreased phosphorylation of PKD was not due to diminished PKD expression (data not shown). To determine whether T cells deficient in ADAP share this reduced PKD phosphorylation phenotype, we evaluated the PKD phosphorylation status in JDAP [an ADAP-deficient Jurkat mutant (29)] and ADAP^−/− T cells. Compared with WT Jurkat T cells, phosphorylation of PKD in JDAP cells is diminished (Fig. 5B, left panel). ADAP^−/− primary T cells also have an overall decreased magnitude of PKD phosphorylation, but prolonged phosphorylation at the autophosphorylation site is frequently seen (Fig. 5B, right panel). Although the impact of this pattern of phosphorylation on T cell activation is unclear, it is not reflective of differences in total PKD levels between WT and ADAP^−/− T cells (data not shown). These data demonstrate that normal PKD phosphorylation is dependent on the SLP-76 SH2 domain and ADAP; however, the mechanism by which these mutations regulate PKD may be different given their distinct pattern of PKD phosphorylation.
and ADAP−/− mice prompted us to interrogate the NF-κB pathway in RK mutant T cells. One prerequisite step in the activation of NF-κB is the phosphorylation and subsequent ubiquitination and degradation of IkBα. IkBα phosphorylation after TCR and CD28 stimulation is robust in WT SLP-76 J14 cells but greatly impaired in the RK mutant SLP-76 J14 cells and nearly absent in vector control cells (Fig. 6A, left panel). T cells from cRK mice also show greatly defective induction of IkBα phosphorylation compared with that of cHet T cells (Fig. 6A, right panel). Consistent with the previous report (37), primary T cells from ADAP−/− mice (and ADAP) also fail to phosphorylate IkBα fully upon TCR stimulation (Fig. 6B). Thus, similar to ADAP−/− T cells, the SH2 domain of SLP-76 is important for activation of the NF-κB pathway; however, our studies cannot rule out separate mechanisms for how ADAP and the SLP-76 SH2 domain couple to this signaling pathway.

Serine phosphorylation of SLP-76 by HPK1 requires the SLP-76 SH2 domain

In addition to ADAP, the SLP-76 SH2 domain associates with HPK1 and is required for full HPK1 activation (12). HPK1 has been defined as both a positive and negative regulator of T cell activation and was recently implicated as a negative regulator of SLP-76 signaling via phosphorylation of SLP-76 S376 (38). To determine if phosphorylation at this site requires a functional SLP-76 SH2 domain, lysates from control and RK mutant T cells were probed using antiserum that detects phosphorylation of SLP-76 at S376. S376 was robustly phosphorylated in WT SLP-76 cells but completely absent when the SLP-76 SH2 domain was mutated (Fig. 7A). To determine whether this HPK1 function is differentially regulated in RK mutant T cells versus ADAP-deficient T cells, we analyzed SLP-76 S376 phosphorylation in ADAP−/− cells. In contrast to RK T cells, S376 phosphorylation in JDAP cells was similar to that seen in WT Jurkat cells (Fig. 7B). This site was also phosphorylated in primary ADAP−/− T cells, although to a slightly reduced level compared with that of WT T cells (Fig. 7B). Differential phosphorylation of SLP-76 S376, and thereby HPK1 function, may contribute to the differences in T cell activation observed between cRK and ADAP−/− mice.

Discussion

Initial studies investigating the importance of the SLP-76 SH2 domain in T cell biology suggested that this domain was not critical for thymocyte development (24). SLP-76−/− mice reconstituted with the SLP-76 RK mutation in the T cell compartment revealed normal thymic cellularity with nearly normal SP T cell development. However, peripheral T cells in these mice did demonstrate diminished T cell proliferation and CD25 upregulation in response to TCR ligation (23, 24). Using cRK mice, we now show that the SH2 domain of SLP-76 is required for SP differentiation, and when cRK mice are mated to TCR transgenic mice, a striking defect in positive selection is revealed. One possible explanation for the differences seen in these two systems may stem from the timing of WT SLP-76 deletion. In the transgenic system, thymocytes use the mutant form of SLP-76 to progress from DN3 to the DP stage, thus potentially selecting for DP cells with altered TCR signaling abilities and masking defects in the DP to SP transition that would be otherwise apparent. WT SLP-76 is present in the conditional system through the β-selection checkpoint, thus supporting normal pre-TCR signaling and progression to the DP stage, thereby allowing for focused analysis of the DP to SP progression.

![FIGURE 6](top panel A, bottom panel A) and ADAP−/− mice. Blots were stripped and reprobed for actin for a loading control. A, WT SLP-76, RK SLP-76, and control J14 transfected cells (left panel, n = 5) and cHet and cRK T cells (right panel, n ≥ 3) were analyzed for IkBα phosphorylation. B, Jurkat and JDAP (left panel, n = 4) and WT and ADAP−/− T cells (right panel, n = 5) were analyzed for IkBα phosphorylation.

![FIGURE 7](top panel B, bottom panel B) The SLP-76 SH2 domain is required for phosphorylation of S376 of SLP-76. T cell lysates from stimulated RK mutant T cells (cell lines, top panel; primary T cells, bottom panel) (A) or ADAP-deficient T cells (cell lines, top panel; primary T cells, bottom panel) (B) were blotted with antiserum against pS376 and Abs to actin and/or total SLP-76 as a loading control (n = 3).
The characteristics of thymic development in RK reconstituted SLP-76^{−/−} and cRK mice are reminiscent of those previously described for ADAP^{−/−} mice (14, 15). Thymocyte selection defects seen in ADAP^{−/−} mice have largely been attributed to defective TCR-induced integrin activation, a process known as inside-out signaling (14, 15). Using T cells from AND transgenic cRK mice, we show that the SH2 domain of SLP-76 is also critical for the formation of Ag-specific T cell–APC conjugates, a function thought to require TCR-driven integrin activation. These data are consistent with our recent demonstration that mutation of the SH2 domain results in decreased TCR-induced adhesion to ICAM and with data from others indicating that the region of ADAP that associates with SLP-76 is important for supporting inside-out signaling and conjugate formation (39, 40). Therefore, defects in thymocyte differentiation seen in cRK mice may be due to the inability of SLP-76 to interact with ADAP.

Analysis of signaling pathways downstream of the TCR revealed that T cells with a mutation in the SLP-76 SH2 domain also share some, but not all, biochemical defects with T cells lacking ADAP. In cell lines and in primary T cells from RK mutant mice, we find that phosphorylation of PKD is greatly diminished at the activation loop sites and autophosphorylation site. The role of PKD in peripheral T cell function has not been fully defined. In T cells, PKD mediates TCR-induced integrin activation through the membrane recruitment and activation of RAP1, a small GTPase important for T cell adhesion (34). Although this study implicated the pleckstrin homology domain of PKD and not its kinase domain in integrin activation, PKD catalytic activity has been implicated in integrin recycling in nonlymphocytes (41). More recently, studies in knockout mice have shown that PKD2 is critical for T cell cytokine production, consistent with earlier experiments in Jurkat cells showing PKD augments AP-1 and NFAT activation (42). Although it is tempting to speculate that diminished PKD activation may partially account for the adhesion and IL-2 production defects observed in RK mutant and ADAP-deficient T cells, additional structure/function analysis and complementation studies will be required to address this question appropriately. We are currently investigating whether SLP-76 and ADAP play a role in PKD localization to the plasma membrane, which is required for activation of PKD, as a possible mechanism for its aberrant activation (43). These experiments may also shed light on the altered kinetics of PKD phosphorylation in ADAP^{−/−} cells.

In addition to defects in T cell–APC conjugates, TCR-driven integrin activation, and PKD phosphorylation, the SLP-76 SH2 domain and ADAP are both required for phosphorylation of IκBα. The simple hypothesis that a SLP-76/ADAP-dependent mechanism of NF-κB activation accounts for functional defects observed in both mice is countered, however, by a previous report suggesting that ADAP regulates NF-κB activation via the assembly of the CBM complex in an SLP-76-independent manner (37). One potential link between the SLP-76 SH2 domain and the NF-κB pathway is through another prominent binding partner of this SLP-76 domain, HPK1. HPK1 activity is dependent upon the SLP-76 SH2 domain (12) (Fig. 7A) and has been reported to activate NF-κB. Initially, HPK1 was shown to activate the IKK complex, leading to phosphorylation of IκB (17). More recently, HPK1 was reported to phosphorylate CARMA1 directly, thus acting as an upstream activator of NF-κB (16). Mutation of the SLP-76 SH2 domain results in loss of HPK1 activity (12), therefore it is possible that failure to activate HPK1 in these cells contributes to their inability to phosphorylate IκB via a pathway that is independent of ADAP.

Recent analysis of HPK1^{−/−} mice reveals that HPK1 also has negative regulatory functions, specifically through its interaction with SLP-76 (19). These data and others show that HPK1 phosphorylation of SLP-76 at S376 induces an association with IκBα and downregulation of TCR signaling through a mechanism that has yet to be defined (19, 38). Additional studies in cell lines show that HPK1 can inhibit AP-1 and NFAT activation and can be cleaved to generate an HPK1 fragment that inhibits NF-κB activation (12, 17, 18, 44). Based on these data and the finding that SLP-76 S376 phosphorylation is largely preserved in ADAP^{−/−} T cells but completely absent in RK mutant T cells, it is surprising that ADAP deficiency does not result in a more severe phenotype compared with that of cRK T cells. Understanding the contribution of HPK1 to the phenotypes observed in cRK and ADAP^{−/−} T cells will require a clearer explanation of the role of HPK1 in TCR-mediated thymocyte selection and activation.

By taking advantage of temporal deletion of WT SLP-76 and combining it with transgenic expression of a SLP-76 mutant, we bypassed the perinatal lethality associated with SLP-76 deficiency, enabling us to investigate rigorously the role of the SH2 domain in T cell development and function. Although many of the functions of this domain appear to be its direct association with the adapter ADAP, we show in this study that the biochemical pathways supported downstream of these two proteins can be differentiated at the level of HPK1 function.

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References


