version (2). Nubbin-binding sites were deleted by cutting the modified enhancer plasmid at unique Sac II and Sph I sites and re-ligating after end repair.
25. E. J. Rulifson, C. A. Micchelli, J. D. Axelrod, N. Perrimon, S. S. Blair, *Nature* **384**, 72 (1996).
26. C. A. Micchelli, E. J. Rulifson, S. S. Blair, *Development* **124**, 1485 (1997).
27. M. Averof and S. M. Cohen, *Nature* **385**, 627 (1997).
28. W. Herr and M. A. Cleary, *Genes Dev.* **9**, 1679 (1995).
Our sites 1 to 3 are similar to other POU sites in having the sequence TT(A/T)T(A/G)A(A/T). Despite their lack of a more extensive consensus sequence, these three sites show near perfect sequence conservation in the corresponding enhancer from *D. virilis* (*23*). We suggest that these sequences are conserved because they represent functionally important binding sites for Nubbin and possibly other proteins.
29. We thank M. Milán for help with *nubbin* clones, M. Ng for constructing the UAS-Nub, R. Nusse and S. Artavanis-Tsakonas for antibodies, F. Pignoni and S. Bray for fly strains, K. Huebner and H. Schoeler for advice on POU proteins, A.-M. Voie for technical assistance, P. Rørth for advice on footprinting, and S. Carroll for communicating unpublished results on mutations of the vestigial intron 2 enhancer.

9 February 1998; accepted 8 June 1998

Uncoupling of Nonreceptor Tyrosine Kinases from PLC-γ1 in an SLP-76–Deficient T Cell

Deborah Yablonski, Michelle R. Kuhne, Theresa Kadlecek, Arthur Weiss*

Activation of nonreceptor protein tyrosine kinases (PTKs) is essential for T cell receptor (TCR) responsiveness; however, the function of individual PTK substrates is often uncertain. A mutant T cell line was isolated that lacked expression of SLP-76 (SH2 domain–containing leukocyte protein of 76 kilodaltons), a hematopoietically expressed adaptor protein and PTK substrate. SLP-76 was not required for TCR-induced tyrosine phosphorylation of most proteins, but was required for optimal tyrosine phosphorylation and activation of phospholipase C-γ1 (PLC-γ1), as well as Ras pathway activation. TCR-inducible gene expression was dependent on SLP-76. Thus, coupling of TCR-regulated PTKs to downstream signaling pathways requires SLP-76.

The T cell antigen receptor (TCR) is coupled to downstream signaling events by nonreceptor protein tyrosine kinases (PTKs) (*1*). The TCR-induced PTKs Lck and ZAP-70 trigger calcium-dependent and Ras-dependent signaling pathways (*2*). TCR-induced calcium flux depends on tyrosine phosphorylation and activation of phospholipase C-γ1 (PLC-γ1), leading to increases in inositol phosphates and intracellular calcium (*3*). Activation of Ras is thought to result from recruitment of a GRB2-SOS complex to the membrane. After TCR stimulation, a membrane-bound adaptor protein, LAT(pp36) (linker for activation of T cells), is heavily tyrosine phosphorylated and subsequently binds GRB2 and PLC-γ1 through their SH2 domains (*4–6*). LAT, therefore, may link PTK activity to activation of both the Ras and calcium pathways.

SLP-76, like LAT, is a GRB2-binding adaptor protein that is tyrosine phosphorylated after TCR stimulation (*7*). SLP-76 comprises a COOH-terminal SH2 domain, a central proline-rich region that binds to the GRB2 SH3 domains, and multiple NH_2-terminal tyrosine phosphorylation sites, which mediate TCR-inducible association of SLP-76 with Vav, a guanine nucleotide exchange factor for Rho-family guanosine triphosphatases (*7–10*). Overexpression of SLP-76 augments TCR-induced transcriptional responses (*8, 9, 11, 12*). However, the mechanism by which overexpression of SLP-76 augments TCR signaling is not understood, and the function of endogenous SLP-76 has been difficult to address.

An SLP-76–deficient T cell, J14, was isolated while screening Jurkat T cell subclones for TCR-inducible expression of the CD69 activation marker (Fig. 1A) (*13*). CD69 is induced in a Ras-dependent manner following stimulation with anti-TCR or with phorbol myristate acetate (PMA) (*14*). Clone J14 lacked TCR-inducible expression of CD69, despite normal TCR expression and normal PMA-induced up-regulation of CD69 (Fig. 1A). This phenotype suggested that the clone is defective at a proximal step in the TCR pathway leading to Ras activation.

Initial characterization of J14 revealed that it lacked SLP-76 protein (Fig. 1B). Numerous other signaling proteins examined were present, including Vav, ZAP-70, PLC-γ1, Erk2, Cbl, Pak1, LAT, Itk, and Nck [(*15*) and below]. Whereas SLP-76 was detected in anti–SLP-76 immune complexes prepared from Jurkat cells, it was undetected in immune complexes prepared from 125 times more J14 cells (*15*).

To determine the significance of the SLP-76 defect, we compared TCR signaling in SLP-76- and vector-transfected subclones of J14 (*16*). SLP-76 transfectants were selected that retained TCR surface expression (*15*) and expressed SLP-76 at wild-type levels. Vector transfectants did not express SLP-76 (Fig. 1B). TCR-induced CD69 expression was restored by expression of SLP-76 (Fig. 1A), showing that the signaling defect of J14 is attributable to the lack of SLP-76.

Northern (RNA) blot analysis revealed that SLP-76 transcripts were reduced in the J14 mutant cells and restored in the SLP-76–reconstituted cells, correlating with expression of the protein (Fig. 1C, top panel). Southern (DNA) blot analysis did not reveal differences in the genomic structure of SLP-76 in J14 cells compared to Jurkat (*15*).

Whereas CD69 induction depends only on the Ras pathway (*14*), TCR-dependent activation of interleukin-2 (IL-2) transcription requires both the TCR-induced Ras and calcium pathways, as well as additional signals, which can be provided by PMA or by the CD28 costimulatory receptor (*17*). After stimulation with anti-TCR and PMA, Jurkat cells up-regulated expression of an IL-2–luciferase reporter, whereas J14 cells did not (Fig. 1D). The J14 subclones stably transfected with SLP-76 responded normally (Fig. 1D). Thus, all of the proteins required for transcriptional activation of IL-2 by the TCR are present in J14, except SLP-76.

In Jurkat T cells, Raji B lymphoblastoid cells plus the superantigen, staphylococcal enterotoxin D (SED), can be used to activate IL-2 transcription. This cell-cell interaction, in which the complex of molecular and associated superantigen major histocompatibility complex stimulates the TCR while other ligands expressed on Raji cells provide costimulation, is a close approximation of in vivo stimulation of a T cell by an antigen-presenting cell (APC). SLP-76–deficient J14 cells did not respond to Raji plus SED, whereas the response was restored in SLP-76–reconstituted cells (Fig. 1D). Thus, SLP-76 is essential for TCR responsiveness to a physiological stimulus delivered during a T cell–APC interaction.

On the basis of the CD69 defect, we hypothesized that TCR-mediated activation of the Ras pathway was defective in J14. Consistent with this interpretation, TCR-mediated, Ras-dependent phosphorylation of Erk2 was substantially reduced in J14 cells relative to Jurkat (Fig. 2A). In contrast, PMA-induced Erk2 activation was equivalent in both cell types (Fig. 2A), and an SLP-76–reconstituted

Departments of Medicine and of Microbiology and Immunology and the Howard Hughes Medical Institute, Box 0795, University of California, San Francisco, San Francisco, CA 94143–0795, USA.

*To whom correspondence should be addressed. E-mail: aweiss@itsa.ucsf.edu

subclone exhibited normal TCR-mediated Erk2 activation (Fig. 2B).

Impaired coupling of the TCR to the Ras pathway in J14 also resulted in reduced activation of an AP-1 transcriptional reporter construct (Fig. 2C). The average TCR-induced activation of the AP-1–luciferase reporter was 8.6 ± 1.3–fold in Jurkat cells compared to 2.6 ± 0.4–fold in J14 cells. As expected, AP-1 activation was restored in the mutant after stimulation with PMA or reexpression of SLP-76. Thus, TCR-mediated activation of both AP-1 and Erk2 was partially reduced in the absence of SLP-76, indicating that SLP-76–deficient cells exhibit an incomplete, but significant defect in TCR-mediated activation of Ras-dependent responses. Further, SLP-76 acts upstream to or independently of Ras, because the defect can be bypassed by stimulation with PMA. Because SLP-76 binds to GRB2, the simplest interpretation is that SLP-76 facilitates efficient signaling through GRB2-SOS; however, other explanations are possible.

To identify other TCR-induced signaling events that depend on SLP-76, we compared tyrosine phosphoproteins induced in J14 and Jurkat cells. In whole-cell lysates, the overall pattern of TCR-induced tyrosine phosphorylation was unchanged, except that the band corresponding to SLP-76 itself was absent in the mutant (Fig. 3A). Immunoprecipitation of specific PTK substrates revealed that TCR-induced tyrosine phosphorylation of Vav, ZAP-70, Itk, and LAT was unaffected by the absence of SLP-76 (Fig. 3B), suggesting that most PTKs are activated normally. In contrast, TCR-induced tyrosine phosphorylation of PLC-γ1 was significantly decreased in SLP-76–deficient cells and was restored after reexpression of SLP-76 (Fig. 3C).

LAT binds to both GRB2 and PLC-γ1 and may thereby initiate signaling through the Ras and calcium pathways, respectively. Because LAT phosphorylation appeared normal, we examined TCR-induced recruitment of both GRB2 and PLC-γ1 to LAT in SLP-76–deficient and SLP-76–reconstituted cells. Recruitment of both signaling molecules to LAT appeared normal in the absence of SLP-76 (Fig. 3D), despite the reduced PLC-γ1 tyrosine phosphorylation.

Consistent with the PLC-γ1 defect, J14 had reduced TCR-induced activation of the inositol phosphate pathway, which was restored in SLP-76–reconstituted cells (Fig. 4A). Furthermore, TCR-induced calcium flux was reduced in SLP-76–deficient cells and was restored to wild-type levels by reexpression of SLP-76 (Fig. 4B).

To test the functional significance of the calcium defect, we examined activation of NFAT (nuclear factor of activated T cells), a composite transcription factor dependent on both the Ras and calcium pathways (*18*). TCR-mediated activation of an NFAT-luciferase reporter plasmid was completely abrogated in SLP-76–deficient J14 cells (Fig. 4C). However, downstream signaling pathways remained intact, because activation of NFAT—using PMA and ionomycin to acti-

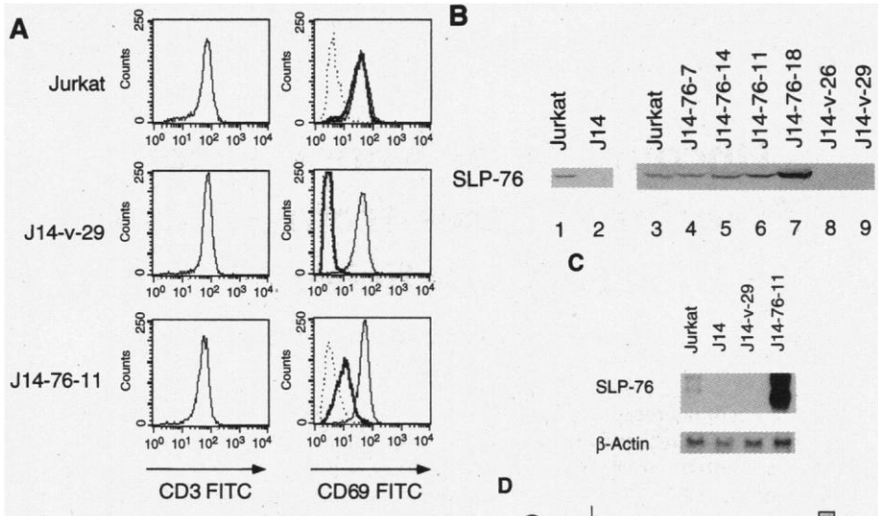
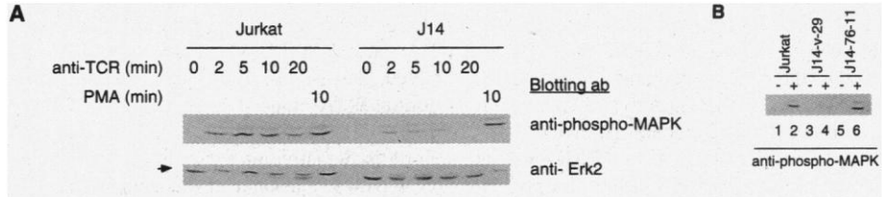


Fig. 1. Characterization of an SLP-76–deficient T cell line. (**A**) We analyzed Jurkat cells (wild type), the SLP-76–deficient cell, J14-v-29, or the SLP-76–reconstituted cell, J14-76-11, by immunofluorescence and flow cytometry, using fluorescein isothiocyanate (FITC)–conjugated (Becton Dickinson) anti-TCR/CD3 (left panels), or anti-CD69 (right panel). CD69 staining was done after a 14-hour incubation with medium (dotted line), monoclonal antibody to TCR, C305 (bold line), or 50 ng/ml PMA (thin line). This experiment is representative of four (*25*). (**B**) SLP-76 protein expression. Total lysates from Jurkat cells (lanes 1 and 3), J14 (lane 2), and independent sub-clones of J14 stably transfected with Flag-tagged SLP-76 (lanes 4 to 7) or a vector control (lanes 8 and 9) were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed by protein immunoblotting using a sheep polyclonal antiserum to residues 136 through 235 of human SLP-76 (*8*). (**C**) SLP-76 mRNA expression. Northern blot analysis of RNA prepared from the indicated cell lines using a [^{32}P]-labeled cDNA probe encompassing the entire open reading frame of SLP-76 (top panel) or a β-actin probe (bottom panel). (**D**) Transcriptional activation of the IL-2 promoter. The indicated cell lines were transiently transfected with an IL-2–luciferase reporter plasmid (*26*), and 20 hours later were stimulated for 6 hours in tissue culture medium alone (white bars) or with 50 ng/ml PMA and immobilized monoclonal antiboy to TCR (C305) (gray bars) or for 8.5 hours with an equal number of Raji B cells alone (lightly striped bars) or supplemented with 300 ng/ml SED (thickly striped bars). Cells were lysed and assayed for luciferase activity as previously described (*9*).



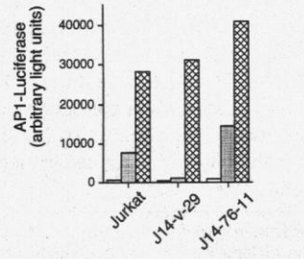


Fig. 2. Effect of SLP-76 on TCR-mediated activation of the Ras pathway. (**A**) Lysates were prepared from Jurkat or J14 cells following stimulation for the indicated time with anti-TCR (C305) or 50 ng/ml PMA (*27*). Total cell lysates were probed by protein immunoblotting with anti-phospho-MAPK (New England Biolabs) (top), which detects phospho-Erk2 (lower band) and phospho-Erk1 (faint, upper band). Lysates were also probed with anti-Erk2 (bottom). The slower migrating band representing activated Erk2 is indicated by an arrow. (**B**) The indicated cells were stimulated with anti-TCR (C305) for 10 min (lanes 2, 4, and 6) or left unstimulated (lanes 1, 3, and 5), and total cell lysates were probed with anti-phosphoMAPK, as in (A). (**C**) The indicated cells were transiently transfected with an AP-1–luciferase reporter plasmid (*28*) and 20 hours later were stimulated for 6 hours and assayed for luciferase activity, as in Fig. 1D. Stimulation was with medium alone (white bars), with immobilized C305 mAb to TCR (gray bars), or with 50 ng/ml PMA (cross-hatched bars).

vate Ras and calcium flux, respectively—was unaffected (Fig. 4C). The impairment of TCR-mediated NFAT activation was more profound than that seen with AP-1, probably reflecting partial inhibition of both the Ras and the calcium pathways. Notably, overexpression of activated Ras (*14*) in SLP-76–deficient cells did not restore TCR responsiveness (Fig. 4D), indicating that the defect of these cells is not confined to events leading to Ras activation. Similarly, overexpression of an activated, calcium-independent form of calcineurin (*19*), the major target of the calcium pathway, did not restore TCR responsiveness. Thus, SLP-76 is required, proximally to the TCR, to achieve functionally sufficient activation of both the Ras and the calcium pathways.

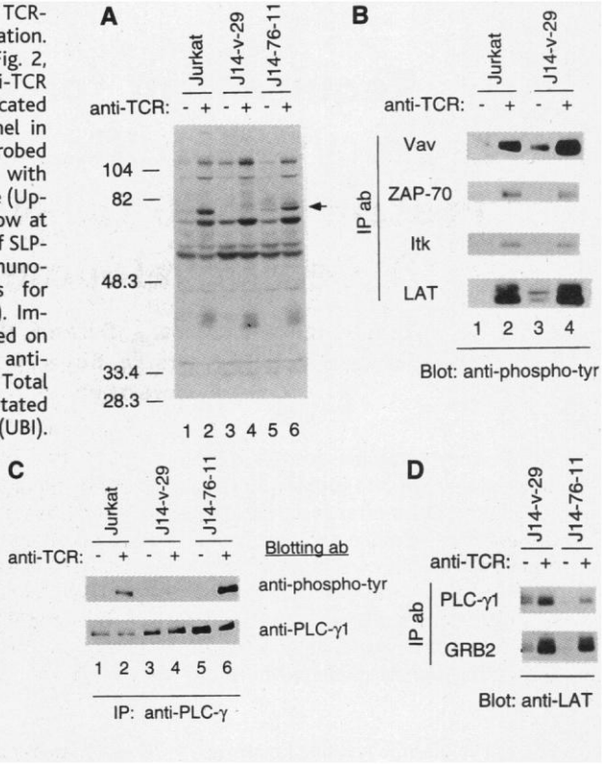


Fig. 3. Effect of SLP-76 on TCR-induced tyrosine phosphorylation. Lysates were prepared as in Fig. 2, following stimulation with anti-TCR (C305) for 2 min, where indicated [or 10 min for the third panel in (B)]. (**A**) Total lysates were probed by protein immunoblotting with 4G10 mAB to phosphotyrosine (Upstate Biotechnology). The arrow at right indicates the migration of SLP-76. (**B**) Cell lysates were immunoprecipitated with antibodies for Vav, ZAP-70, Itk, or LAT (*29*). Immune complexes were resolved on SDS-PAGE and blotted with anti-phosphotyrosine as in (A). (**C**) Total lysates were immunoprecipitated with antibodies to PLC-γ1 (UBI). Immune complexes were resolved on SDS-PAGE and blotted with anti-phosphotyrosine (top) or anti–PLC-γ1 (bottom). (**D**) Lysates were prepared and immunoprecipitated as above, except that Brij97 detergent was substituted for NP-40. Anti–PLC-γ1 or anti-GRB2 (Santa Cruz) immune complexes were resolved on SDS-PAGE and blotted with anti-LAT.

We show a requirement for SLP-76 to mediate TCR signaling and define SLP-76–dependent signaling events. SLP-76 is probably required to increase the strength or duration of TCR signaling through both the Ras and calcium pathways to above the threshold required for a biological response. Consistent with this conclusion are independent observations that SLP-76–deficient mice have a thymic developmental arrest at a checkpoint that requires pre-TCR signal transduction (*20*).

The specific signaling events that were dependent on SLP-76 are also thought to involve LAT. LAT forms TCR-inducible complexes with GRB2 and PLC-γ1 (*5, 6*), thereby linking to the Ras and calcium pathways, and also associates with SLP-76, though this interaction may not be direct (*6, 8*). LAT was inducibly tyrosine phosphorylated in J14; nonetheless, neither PLC-γ1 nor Ras activation resulted. To explain these observations, we suggest that SLP-76 may participate in the formation of a multimolecular signaling complex, nucleated by LAT, and may facilitate signaling events within this complex. Any of a number of SLP-76–associated proteins (*8, 9, 11, 21*) may be recruited to the signaling complex by SLP-76. Vav, a hematopoietically expressed member of the Dbl family of guanine nucleotide exchange factors (*22*), can cooperate with SLP-76 to augment TCR signaling (*9*); furthermore, Vav-deficient thymocytes, like J14, have defective TCR-induced calcium flux and CD69 expression (*23*). Thus, SLP-76 may facilitate transmission of a Vav-mediated signal required for optimal TCR-dependent activation of Ras and calcium flux. Another possibility

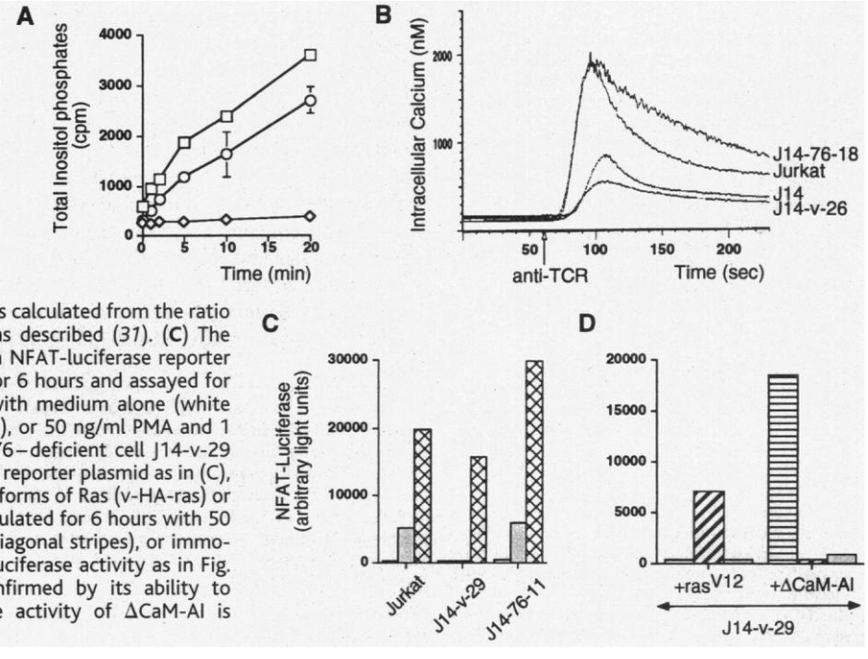


Fig. 4. Impaired inositol phosphate and ras pathway in SLP-76–deficient cells. (**A**) Jurkat T cells (squares), the SLP-76–deficient clone J14-v-29 (diamonds), and the SLP-76–reconstituted clone J14-76-11(circles) were metabolically labeled with [^{3}H]myo-inositol, stimulated with anti-TCR in the presence of LiCl for the indicated time, and total accumulation of inositol phosphates was measured as described (*30*). (**B**) Jurkat T cells, the SLP-76–deficient clones J14 and J14-v-26, and the SLP-76–reconstituted clone J14-76-18 were loaded with the fluorescent calcium indicator dye Indo-1 (Molecular Probes) and stimulated with anti-TCR (C305), and free calcium concentration was calculated from the ratio of fluorescence at 400 and 500 nm wavelength, as described (*31*). (**C**) The indicated cells were transiently transfected with an NFAT-luciferase reporter plasmid (*26*), and 20 hours later were stimulated for 6 hours and assayed for luciferase activity as in Fig. 1D. Stimulation was with medium alone (white bars), immobilized anti-TCR mAb (C305) (gray bars), or 50 ng/ml PMA and 1 μM ionomycin (cross-hatched bars). (**D**) The SLP-76–deficient cell J14-v-29 was transiently transfected with an NFAT-luciferase reporter plasmid as in (C), along with a plasmid encoding constitutively active forms of Ras (v-HA-ras) or calcineurin (ΔCaM-AI), as indicated. Cells were stimulated for 6 hours with 50 ng/ml PMA (horizontal stripes), 1 μM ionomycin (diagonal stripes), or immobilized anti-TCR (gray bars) and were assayed for luciferase activity as in Fig. 1D. The constitutive activity of v-HA-ras is confirmed by its ability to synergize with ionomycin, while the constitutive activity of ΔCaM-AI is confirmed by its synergy with PMA.

is that SLP-76 may recruit a PTK to the complex to mediate phosphorylation of PLC-γ1. Our results provide a basis for understanding the biochemical coupling of SLP-76 to specific signaling events.

References and Notes

1. A. Weiss and D. R. Littman, *Cell* **76**, 263 (1994); A. C. Chan and A. S. Shaw, *Curr. Opin. Immunol.* **8**, 394 (1996); R. L. Wange and L. E. Samelson, *Immunity* **5**, 197 (1996).
2. D. Cantrell, *Annu. Rev. Immmunol.* **14**, 259 (1996); D. Qian and A. Weiss, *Curr. Opin. Cell Biol.* **9**, 205 (1997).
3. S. G. Rhee and K. D. Choi, *J. Biol. Chem.* **267**, 12393 (1992); A. Weiss, G. Koretzky, R. C. Schatzman, T. Kadlecek, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5484 (1991); C. H. June *et al.*, *ibid.* **87**, 7722 (1990); T. Mustelin, K. M. Coggeshall, N. Isakov, A. Altman, *Science* **247**, 1584 (1990); D. J. Park, H. W. Rho, S. G. Rhee, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5453 (1991); J. P. Secrist, L. Karnitz, R. T. Abraham, *J. Biol. Chem.* **266**, 12135 (1991).
4. L. Buday, S. E. Egan, P. Rodriguez-Viciana, D. A. Cantrell, J. Downward, *J. Biol. Chem.* **269**, 9019 (1994); D. G. Motto, M. A. Musci, S. E. Ross, G. A. Koretzky, *Mol. Cell. Biol.* **16**, 2823 (1996).
5. M. Sieh, A. Batzer, J. Schlessinger, A. Weiss, *Mol. Cell. Biol.* **14**, 4435 (1994).
6. W. Zhang, J. Sloan-Lancaster, J. Kitchen, R. P. Trible, L. E. Samelson, *Cell* **92**, 83 (1998).
7. J. K. Jackman *et al.*, *J. Biol. Chem.* **270**, 7029 (1995).
8. D. G. Motto, S. E. Ross, J. Wu, L. R. Hendricks-Taylor, G. A. Koretzky, *J. Exp. Med.* **183**, 1937 (1996).
9. J. Wu, D. G. Motto, G. A. Koretzky, A. Weiss, *Immunity* **4**, 593 (1996).
10. N. Fang, D. G. Motto, S. E. Ross, G. A. Koretzky, *J. Immunol.* **157**, 3769 (1996).
11. J. B. Wardenburg *et al.*, *J. Biol. Chem.* **271**, 19641 (1996).
12. M. A. Musci, D. G. Motto, S. E. Ross, N. Fang, G. A. Koretzky, *J. Immunol.* **159**, 1639 (1997).
13. Jurkat cells were transfected with an episomal vector and clones selected in hygromycin-containing medium. Clone J14 lacked TCR-mediated CD69 up-regulation, independently of the episomal vector, which was subsequently lost during growth in nonselective media. Absence of integration of the episomal DNA was confirmed by Southern blotting.
14. D. D'Ambrosio, D. A. Cantrell, L. Frati, A. Santoni, R. Testi, *Eur. J. Immunol.* **24**, 616 (1994).
15. D. Yablonski, M. R. Kuhner, T. Kadlecek, A. Weiss, data not shown.
16. Flag-tagged SLP-76 (Sal I–Xba I fragment) (*19*) was subcloned into pAWneo3′, which bears the neomycin-selectable marker. J14 cells were stably transfected with the resulting plasmid or the pAWneo3′ vector by standard procedures.
17. J. D. Fraser, D. Straus, A. Weiss, *Immunol. Today* **14**, 357 (1993); A. Rao, C. Luo, P. G. Hogan, *Annu. Rev. Immunol.* **15**, 707 (1997).
18. M. Woodrow, N. A. Clipstone, D. Cantrell, *J. Exp. Med.* **178**, 1517 (1993).
19. S. J. O'Keefe, J. Tamura, R. L. Kincaid, M. J. Tocci, E. A. O'Neill, *Nature* **357**, 692 (1992).
20. J. L. Clements *et al.*, *Science* **281**, 416 (1998).
21. A. J. da Silva *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7493 (1997); M. A. Musci *et al.*, *J. Biol. Chem.* **272**, 11674 (1997).
22. X. R. Bustelo, *Crit. Rev. Oncog.* **7**, 65 (1996).
23. M. Turner *et al.*, *Immunity* **7**, 451 (1997); R. Zhang, F. W. Alt, L. Davidson, S. H. Orkin, W. Swat, *Nature* **374**, 471 (1995); K.-D. Fischer *et al.*, *ibid.*, p. 474.
24. A. C. Chan, I. Makio, C. W. Turck, A. Weiss, *Cell* **71**, 649 (1992); X. C. Liao and D. R. Littman, *Immunity* **3**, 757 (1995).
25. No significant differences were found between J14 and the vector-transfected subclones nor between different SLP-76–transfected subclones in any of the assays described; therefore, representative results are shown throughout.
26. The NFAT and IL-2–luciferase reporter constructs were provided by G. Crabtree (Stanford University).
27. Cells were mock stimulated with phosphate-buffered saline (no stimulation) or stimulated with C305 ascites (1:500), rapidly collected, and lysed in NP-40 lysis buffer as described (*9*). Whole-cell lysates or washed immune complexes were resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P (Millipore) and probed with primary and secondary antibodies as described (*9*).
28. V. S. Shapiro, M. N. Mollenauer, W. C. Greene, A. Weiss, *J. Exp. Med.* **184**, 1663 (1996).
29. Antibodies to ZAP-70, Itk, and LAT have been described (*6*, *24*).
30. J. B. Imboden and J. D. Stobo, *J. Exp. Med.* **161**, 446 (1985).
31. G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **260**, 3440 (1985).

32. We thank G. Koretzky for providing us with antiserum to human SLP-76 and the SLP-76 cDNA and for communicating results before publication, L. Samelson for the antiserum to LAT, N. van Oers for the monoclonal antibodies to Vav, C. Liao for the antiserum to Itk, and the Weiss lab for their discussions and comments. Supported in part by a grant from the National Cancer Institute, CA72531.

10 April 1998; accepted 19 June 1998

Requirement for the Leukocyte-Specific Adapter Protein SLP-76 for Normal T Cell Development

James L. Clements, Baoli Yang, Susan E. Ross-Barta, Steve L. Eliason, Ronald F. Hrstka, Roger A. Williamson, Gary A. Koretzky*

The leukocyte-specific adapter molecule SLP-76 (Src homology 2 domain–containing leukocyte protein of 76 kilodaltons) is rapidly phosphorylated on tyrosine residues after receptor ligation in several hematopoietically derived cell types. Mice made deficient for SLP-76 expression contained no peripheral T cells as a result of an early block in thymopoiesis. Macrophage and natural killer cell compartments were intact in SLP-76–deficient mice, despite SLP-76 expression in these lineages in wild-type mice. Thus, the SLP-76 adapter protein is required for normal thymocyte development and plays a crucial role in translating signals mediated by pre–T cell receptors into distal biochemical events.

Activation of cytoplasmic tyrosine kinase activity is required for T cell receptor (TCR)–dependent lymphocyte activation (*1*). Adapter proteins serve as substrates for these kinases and as such may function to couple the TCR with downstream signaling events (*2*–*6*). SLP-76 is a hematopoietic cell–specific adapter protein that is phosphorylated rapidly on NH_2-terminal tyrosine residues after TCR ligation (*3*), providing a binding site for the Src homology 2 (SH2) domain of Vav (*7*). SLP-76 also contains a central proline-rich region that associates constitutively with the SH3 domains of Grb2 (*8*). In addition, SLP-76 has a COOH-terminal SH2 domain that inducibly associates with SLAP-130 (SLP-76–associated phosphoprotein of 130 kD) and an unidentified 62-kD tyrosine phosphoprotein (*5*, *8*, *9*). The ability of SLP-76 to augment TCR-dependent nuclear factor of activated T cells (NFAT) activation when transiently overexpressed in a T cell line is dependent on the presence of each of these domains, suggesting that the association between SLP-76 and at least a subset of these molecules is required for optimal function (*10*).

In mice, SLP-76 expression is restricted to T lymphocytes, macrophages, and natural killer (NK) cells (*11*). SLP-76 is developmentally regulated during thymopoiesis, with highest expression found at stages of development that coincide with pre-TCR–dependent selection and maturation from a $CD4^+CD8^+$ phenotype to a $CD4^+$ or $CD8^+$ thymocyte (*11*). To define the role of SLP-76 in murine T cell development and function, we generated an SLP-76–deficient mouse strain through targeted disruption of the SLP-76 genomic locus (*12*) (Fig. 1). About 360 base pairs (bp) of the SLP-76 genomic locus, including 145 bp of the first exon containing the translational start site, were replaced with a neomycin resistance cassette in

J. L. Clements, S. E. Ross-Barta, S. L. Eliason, Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, IA 52242, USA. B. Yang, R. F. Hrstka, R. A. Williamson, Department of Obstetrics and Gynecology, University of Iowa College of Medicine, Iowa City, IA 52242, USA. G. A. Koretzky, Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, IA 52242, USA.

*To whom correspondence should be addressed. E-mail: gary-koretzky@uiowa.edu