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

the reverse transcriptional orientation. In a properly targeted allele, the wild-type 14-kb Bam HI fragment is converted to a 10-kb fragment as a result of the incorporation of a novel Bam HI site contained within the targeting vector. Of 85 neomycin- and gancyclovir-resistant embryonic stem (ES) cell clones analyzed, we found 6 (7%) to contain a properly targeted SLP-76 allele. Two of these clones were microinjected into C57BL/6 blastocysts, and chimeric mice were then bred to wild-type C57BL/6 mice. Germ line transmission was confirmed by Southern blot analysis of tail genomic DNA. Heterozygous mice were then mated to obtain homozygous SLP-76–deficient mice.

As determined by Southern blot analysis of tail genomic DNA obtained from heterozygous matings (Fig. 1B), the frequency of SLP-76–deficient (−/−) mice was ~7%, whereas wild-type (+/+) and heterozygous (+/−) mice represented 29 and 64%, respectively, of the total progeny screened (N = 135). Despite the low frequency of SLP-$76^{-/-}$ mice, they showed no major developmental abnormalities at any time during the first 10 weeks of life. To verify the absence of SLP-76 gene products in SLP-$76^{-/-}$ mice, we used reverse transcriptase–polymerase chain reaction (RT-PCR) to analyze SLP-76 expression in samples obtained from control, +/−, and −/− mice (*13*). Whereas two independent sets of PCR primers specific for SLP-76 amplified the appropriate cDNA from control and SLP-$76^{+/-}$ mice, no such amplification product was detected in cDNA derived from SLP-$76^{-/-}$ mice (Fig. 1C). Furthermore, SLP-76 protein was not detectable in SLP-$76^{-/-}$ splenocytes by protein immunoblot analysis or intracellular staining with a fluorochrome-conjugated, affinity-purified, SLP-76–specific sera (*14*).

Upon dissection, it was apparent that lymph nodes from SLP-$76^{-/-}$ mice were smaller than those observed in +/+ or +/− mice. In contrast, spleens from SLP-$76^{-/-}$ mice were enlarged, resulting in a two- to threefold increase in cell yield. Fluorescence-activated cell sorter (FACS) analysis of cell populations in the spleen revealed a complete lack of T cells ($CD3^+$, $CD4^+$, or $CD8^+$) in SLP-$76^{-/-}$ mice (*15*) (Fig. 2A). The minor population of γδ-TCR^+ lymphocytes was also absent from the spleen and liver (*14*). The B cell ($B220^+$) compartment was intact in the spleen from SLP-$76^{-/-}$ mice, consistent with our observation that primary murine B cells do not express detectable amounts of SLP-76 (*11*). Like the spleen, no T lymphocytes were detected in peripheral blood obtained from SLP-$76^{-/-}$ mice, whereas normal percentages of B lymphocytes were present (*14*). Spleens from SLP-$76^{-/-}$ mice contained macrophages ($Mac\text{-}1^+$) and at least a subset of NK ($DX5^+CD3^-$) lymphocytes (Fig. 2A), despite expression of SLP-76 in these cell types in

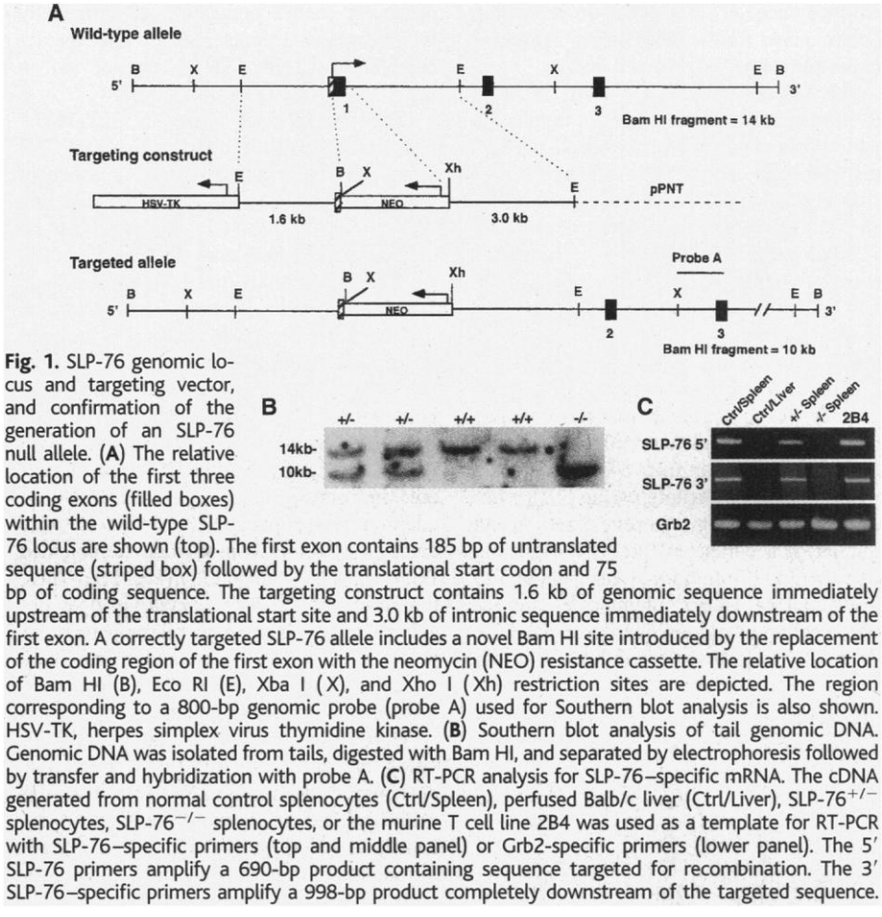

Fig. 1. SLP-76 genomic locus and targeting vector, and confirmation of the generation of an SLP-76 null allele. (**A**) The relative location of the first three coding exons (filled boxes) within the wild-type SLP-76 locus are shown (top). The first exon contains 185 bp of untranslated sequence (striped box) followed by the translational start codon and 75 bp of coding sequence. The targeting construct contains 1.6 kb of genomic sequence immediately upstream of the translational start site and 3.0 kb of intronic sequence immediately downstream of the first exon. A correctly targeted SLP-76 allele includes a novel Bam HI site introduced by the replacement of the coding region of the first exon with the neomycin (NEO) resistance cassette. The relative location of Bam HI (B), Eco RI (E), Xba I (X), and Xho I (Xh) restriction sites are depicted. The region corresponding to a 800-bp genomic probe (probe A) used for Southern blot analysis is also shown. HSV-TK, herpes simplex virus thymidine kinase. (**B**) Southern blot analysis of tail genomic DNA. Genomic DNA was isolated from tails, digested with Bam HI, and separated by electrophoresis followed by transfer and hybridization with probe A. (**C**) RT-PCR analysis for SLP-76–specific mRNA. The cDNA generated from normal control splenocytes (Ctrl/Spleen), perfused Balb/c liver (Ctrl/Liver), SLP-$76^{+/-}$ splenocytes, SLP-$76^{-/-}$ splenocytes, or the murine T cell line 2B4 was used as a template for RT-PCR with SLP-76–specific primers (top and middle panel) or Grb2-specific primers (lower panel). The 5′ SLP-76 primers amplify a 690-bp product containing sequence targeted for recombination. The 3′ SLP-76–specific primers amplify a 998-bp product completely downstream of the targeted sequence.

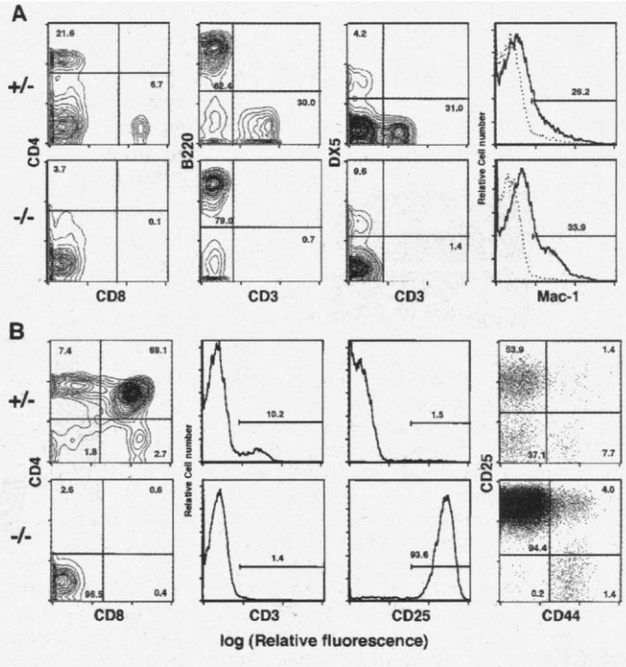

Fig. 2. SLP-76–deficient mice contain no peripheral T cells because of an early block in thymopoiesis. (**A**) Splenocytes were obtained from 10-week-old SLP-76 +/− or −/− mice by density gradient centrifugation and surface stained with antibodies to the indicated proteins. Only those cells with forward and side scatter characteristics indicative of lymphocytes were included in the analysis. In these and subsequent experiments, staining with isotype-matched, nonspecific, fluorochrome-conjugated control antibodies was performed to establish background staining levels (*14*). The percentage of cells in each quadrant or gate is shown. (**B**) Thymocytes were isolated from 10-week-old SLP-76 +/− or −/− mice and stained with fluorochrome-conjugated antibodies to the indicated proteins. For the last panel in each group, cells that stained positive with FITC-conjugated antibodies specific for CD3, CD4, and CD8 were excluded from analysis, and only those cells that stained positive for Thy-1.2 were analyzed for expression of CD44 and CD25 allowing for the exclusion of nonthymocytes, which could potentially contaminate the preparation. Virtually 100% of the SLP-$76^{-/-}$ thymocytes were $CD3^-CD4^-CD8^-$. The percentage of cells in each quadrant or gate is shown.

wild-type mice (*11*). Thus, SLP-76 expression is not required for the development of macrophages and the DX5$^+$ NK cell lineage.

The lack of peripheral T cells in SLP-76$^{-/-}$ mice suggested a defect in thymocyte maturation and development. Indeed, the thymus was small and difficult to identify in 6- to 10-week-old SLP-76$^{-/-}$ mice. As a result, cell yield was reduced by a factor of about 10 to 20 compared with +/+ or +/− littermate controls (*14*). The reduced cell number in the SLP-76$^{-/-}$ thymus was the result of an early block in thymocyte development, as shown by the complete lack of CD4$^+$CD8$^+$, CD4$^+$, or CD8$^+$ thymocyte populations (Fig. 2B). Closer inspection of the thymocytes obtained from SLP-76$^{-/-}$ mice revealed a specific block at the transition from a CD44$^-$CD25$^+$ to CD44$^-$CD25$^-$ phenotype (Fig. 2B), a developmental step that requires expression and function of a competent pre-TCR signaling complex (*16, 17*). In normal mice, about 5 to 10% of CD44$^-$CD25$^+$ thymocytes are actively cycling as a consequence of pre-TCR ligation (*17*). However, we did not find evidence for such a population as defined by forward scatter characteristics within the accumulating CD44$^-$CD25$^+$ thymocytes in SLP-76$^{-/-}$ mice (*14*).

The maturational arrest in SLP-76$^{-/-}$ mice could be due to either a lack of TCRβ chain gene rearrangement and subsequent pre-TCR expression or defective signaling initiated by the pre-TCR complex (*18*). To determine if the immature thymocytes isolated from SLP-76$^{-/-}$ mice could rearrange TCRβ chain gene segments, we performed PCR and Southern blot analysis to look for specific recombination events (*19*) (Fig. 3A). We detected identical rearrangement products in thymocytes obtained from both SLP-76$^{+/-}$ and SLP-76$^{-/-}$ mice. Only the germ line (GL) $D_\beta2$-$J_\beta2$ configuration was detectable in control ES cell genomic DNA. In addition, transcripts specific for preTα were detected by RT-PCR in thymocytes obtained from SLP-76$^{-/-}$ mice (*13*) (Fig. 3B). Thus, the absence of SLP-76 expression does not affect TCRβ chain gene rearrangement or preTα mRNA expression and more likely affects the ability of the pre-TCR complex to transduce maturational signals.

Given the observation that B cells develop normally in SLP-76$^{-/-}$ mice, we determined the proliferative capacity of B cells isolated from these animals in response to several polyclonal stimuli. In addition, we measured basal IgM concentrations in sera obtained from SLP-76 +/+, +/−, and −/− mice (*20*). Splenocytes isolated from both +/− and −/− mice responded comparably to polyclonal B cell stimuli (LPS and CD40 ligation) as assayed by thymidine incorporation after 48 or 72 hours in culture (Fig. 4A). The increased proliferative capacity of splenocytes isolated from SLP-76$^{-/-}$ mice in response to CD40 ligation and LPS is likely due to the higher proportion of B cells in these preparations. As expected, splenocytes from SLP-76$^{-/-}$ mice failed to respond to plate-bound anti-CD3ε because of the lack of peripheral T cells. Comparable concentrations of IgM were detected in the serum from SLP-76 +/+, +/−, and −/− mice (Fig. 4B). Thus, the B lymphocytes that develop in SLP-76$^{-/-}$ mice retain the ability to proliferate in response to polyclonal stimuli and secrete normal amounts of basal IgM.

Developing thymocytes are subjected to a rigorous receptor-dependent selection process, whereas other SLP-76–expressing hematopoietic cell lineages mature in the absence of selection. This may explain why SLP-76 deficiency has such a profound effect on the T cell compartment but does not affect macrophage and NK cell development. Early maturation events in the thymus are governed by a pre-TCR signaling complex composed of a properly rearranged TCR β chain and a surrogate α chain, preTα (*21*). Upon ligation, the pre-TCR initiates biochemical signals similar to those elicited upon ligation of a mature TCR complex, including activation of cytoplasmic tyrosine kinases (*22*). The best candidate for the kinase responsible for tyrosine phosphorylation of SLP-76 after TCR ligation is the Syk family tyrosine kinase ZAP-70 (*23*). ZAP-70 deficiency in mice results in a comparatively mild developmental block at the CD4$^+$CD8$^+$ to single-positive transition (*24*). Arrested development at the CD44$^-$CD25$^+$ stage of thymopoiesis is only realized when both ZAP-70 and Syk are deficient (*25*). Similarly, arrest at the CD44$^-$CD25$^+$ developmental checkpoint is only observed when both Lck and Fyn are absent (*26*). Thus, unlike the Src and Syk family tyrosine kinases, there appears to be no redundancy at the level of SLP-76 function in pre-TCR–dependent signaling pathways and during thymocyte development.

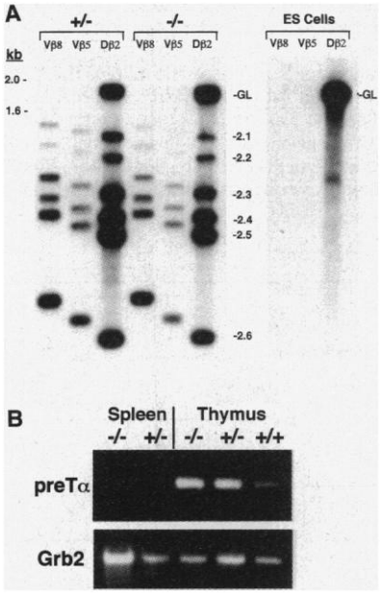

Fig. 3. Detection of TCR β chain rearrangement and preTα mRNA expression in immature thymocytes obtained from SLP-76$^{-/-}$ mice. (**A**) Genomic DNA obtained from SLP-76 +/− or −/− thymocytes was used as a template for PCR with the indicated 5′ sense primer and a common antisense primer located just downstream of the $J_\beta2.6$ gene segment. PCR products were resolved by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with a oligomer probe specific for the $J_\beta2.6$ gene segment. Specific $D_\beta2$ to $J_\beta2$ rearrangement products are noted. An ES cell line was used as a source of nonrearranged control genomic DNA. (**B**) The cDNA generated from total RNA isolated from thymus and spleen from the indicated mice was used as a template in PCR with primers specific for preTα or Grb2.

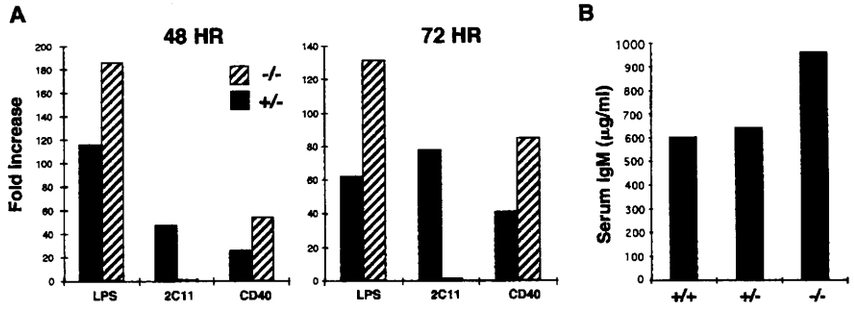

Fig. 4. B cells from SLP-76$^{-/-}$ mice proliferate in response to polyclonal stimuli and generate normal amounts of IgM. (**A**) Splenocytes were isolated from SLP-76$^{+/-}$ mice (solid bar) or −/− mice (hatched bar) and cultured in triplicate in media alone or stimulated with the indicated reagents for 48 or 72 hours (HR). After culture, proliferation was determined by measurement of [^{3}H]thymidine incorporation during the last 4 hours of culture. The fold increase in proliferation was calculated by dividing the counts per minute obtained under conditions of stimulation by the counts per minute from nonstimulated cultures. (**B**) Sera were obtained from SLP-76 +/+, +/−, and −/− mice, and concentrations of circulating IgM were determined by enzyme-linked immunosorbent assay.

References and Notes

1. C. H. June *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7722 (1990); D. B. Straus and A. Weiss, *Cell* **70**, 585 (1992); K. Chu and D. R. Littman, *J. Biol. Chem.* **269**, 24095 (1994).
2. C. T. Baldari *et al.*, *Oncogene* **10**, 1141 (1995).
3. J. Jackman *et al.*, *J. Biol. Chem.* **270**, 7029 (1995).
4. J. A. Donovan, R. L. Wange, W. Y. Langdon, L. E. Samelson, *ibid.* **269**, 22921 (1994).
5. M. Musci *et al.*, *ibid.* **272**, 11674 (1997).
6. W. Zhang, J. Sloan-Lancaster, J. Kitchen, R. P. Trible, L. E. Samelson, *Cell* **92**, 83 (1998).
7. J. Wu, D. G. Motto, G. A. Koretzky, A. Weiss, *Immunity* **4**, 593 (1996); H. Onodera, D. G. Motto, G. A. Koretzky, D. M. Rothstein, *J. Biol. Chem.* **271**, 22225 (1996); L. Tuosto, F. Michel, O. Acuto, *J. Exp. Med.* **184**, 1161 (1996).
8. D. G. Motto, S. E. Ross, J. Wu, L. R. Hendricks-Taylor, G. A. Koretzky, *J. Exp. Med.* **183**, 1937 (1996).
9. A. J. da Silva *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7493 (1997).
10. M. A. Musci, D. G. Motto, S. E. Ross, N. Fang, G. A. Koretzky, *J. Immunol.* **159**, 1639 (1997).
11. J. L. Clements, S. E. Ross-Barta, G. A. Koretzky, in preparation. We have also detected SLP-76 expression in the NK cell lineage in normal mice. However, the intensity of SLP-76 staining observed in NK cells is ~50% of that observed in resting T cells.
12. A pair of polymerase chain reaction (PCR) primers (forward, 5′-TCGACTCGATCAGACCTGAAGATGAAG-3′; reverse 5′-GCTTCTGTCTATTGATGGAGCAGG-3′) were designed to amplify a 320-bp product composed of SLP-76 genomic sequence including an exon encoding amino acids 208 to 229, the following 96-bp intron, and a portion of the next exon encoding amino acids 230 to 257. These primers were used to obtain a commercial P1 murine SLP-76 genomic library (strain 129/SVJ, Genome Systems, St. Louis). To generate a targeting vector specific for the SLP-76 genomic locus, we amplified from the P1 clone a 1.6-kb PCR fragment comprising sequence immediately upstream of the SLP-76 translational start site and subcloned it into the Bam HI and Eco RI restriction sites in the pPNT parental vector. Next, a 3-kb Xho I–flanked, PCR-amplified product composed of SLP-76 intronic sequence located between exon 1 and exon 2 was subcloned into the Xho I site of pPNT. The targeting construct was then transfected by electroporation into ES cells. G418- and gancyclovir-resistant colonies were isolated and screened by Southern (DNA) blot analysis for a correctly targeted SLP-76 allele. Targeted clones were microinjected into blastocyts obtained from C57/BL6 mice, which were then transferred to pseudopregnant CD-1 recipient mice. Chimeric offspring were mated with wild-type C57/B6 mice, and heterozygous offspring were identified by Southern blot analysis of tail genomic DNA.
13. Total RNA was prepared from freshly isolated thymocytes, splenocytes, or the indicated cell line with Stat-60 (Tel-Test) according to the manufacturer's protocol. Approximately 2 μg of total RNA was used for cDNA synthesis in a 20-μl reaction mixture containing 50 ng of oligo(dT) primers (Life Technologies) and 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies). A 5-μl sample of the cDNA synthesis reaction was then used for PCR with primers specific for 5′ SLP-76 sequence (forward, 5′-TTTCGCTCAGAGGTCCTAGCC-3′; reverse, 5′-GCTTCTGTCTATTGATGGAGCAGG-3′), 3′ SLP-76 sequence (forward, 5′-TCGGATCCCAGGGCCTCTCA-AGGCCTCCTC-3′; reverse, 5′-GTCGGATCCTAAGTG-CTCGGCGATTTG-3′), preTα (forward, 5′-GCTGCTT-CTGGGCGTCAGG-3′; reverse, 5′-CAGGCCTGGTGT-GACAGACC-3′), or Grb2 as a control (forward, 5′-GGCGGATCCGAAGCCATCGCCAAATATGACTTC-3′; reverse, 5′-GGGAATTCAGACGTTCCGGTTCACGGG-GGTGAC-3′).
14. J. L. Clements *et al.*, data not shown.
15. The following antibodies were used for FACS analysis: Rat antibodies to mouse CD11b (Mac-1, M1/70.15.11.5.HL), CD4 (GK1.5), CD8 (53.6.72), CD44 (9F3), B220 (6B2), and CD25 (7D4) were partially purified from serum-free supernatants (HB101) and were conjugated where indicated with fluorescein isothiocyanate (FITC), biotin, Cyanine 5-18, or phycoerythrin (PE) using standard procedures. The FITC-conjugated rat antibody to mouse pan (anti–mouse pan) specific for NK cells (DX-5), FITC-conjugated hamster anti–mouse CD3ε (145-2C11), PE-conjugated rat anti–mouse Thy-1.2 (53-2.1), and biotin-conjugated hamster anti–mouse TCRβ (H57-597) were purchased from Pharmingen. All samples were analyzed with a Coulter EPICS 753 instrument, and FACS data were analyzed with Flojo version 2.3.3 software (Treestar).
16. H. J. Fehling, A. Krotkova, C. Saint-Ruf, H. von Boehmer, *Nature* **375**, 795 (1995).
17. E. S. Hoffman *et al.*, *Genes Dev.* **10**, 948 (1996).
18. P. Mombaerts *et al.*, *Cell* **68**, 869 (1992); Y. Shinkai *et al.*, *ibid.*, p. 855 (1992); T. Molina *et al.*, *Nature* **357**, 161 (1992).
19. Genomic DNA was prepared from SLP-76 +/− or −/− thymocytes and the ES cell line 129. We performed PCR using primers and conditions previously described (*27*) in a total volume of 50 μl. Next, 15 μl of the reaction products was resolved by electrophoresis in a 1.5% agarose gel, transferred to a nylon membrane, and probed with a radiolabeled oligomer corresponding to the $J_\beta 2.6$ gene segment (5′-GAA-CAGTACTTCGGTCCCGGCACCAGGCTC-3′) in a 6× standard saline citrate (SSC)–based hybridization buffer.
20. For proliferation assays, 0.2×10^6 splenocytes from SLP-76 +/− or −/− mice were incubated in 200 μl of RPMI supplemented with 10% fetal bovine serum and 2-ME or stimulated with lipopolysaccharide (10 μg/ml), anti-CD40 (Pharmingen, 1 μg/ml), or plate-bound anti-CD3ε [145-2C11, plated in phosphate-buffered saline (PBS) at 8 μg/ml]. After 48 or 72 hours of culture, cells were pulsed with 1 μCi of [^{3}H]thymidine (Amersham) and incubated for an additional 4 hours before they were harvested and analyzed by liquid scintillation. To quantitate concentrations of circulating immunoglobulin M (IgM), we serially diluted sera obtained from SLP-76 +/+, +/−, and −/− mice and incubated the sera in the presence of a plate-bound, isotype-specific capture antibody (μ-chain specific, Jackson Laboratories). Plates were washed and incubated with an alkaline phosphatase–conjugated antibody specific for mouse Ig (Pharmingen). After extensive washing, bound antibody was detected with p-PNP substrate (Pharmingen), and absorbance was determined at 405 nm. The IgM concentrations were determined by comparison with an IgM standard curve.
21. C. Saint-Ruf *et al.*, *Science* **266**, 1208 (1994).
22. N. S. van Oers, H. von Boehmer, A. Weiss, *J. Exp. Med.* **182**, 1585 (1995).
23. M. Raab, A. J. da Silva, P. R. Findell, C. E. Rudd, *Immunity* **6**, 155 (1997).
24. I. Negishi *et al.*, *Nature* **376**, 435 (1995).
25. A. M. Cheng *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9797 (1997).
26. N. S. van Oers, B. Lowin-Kropf, D. Finlay, K. Connolly, A. Weiss, *Immunity* **5**, 429 (1996).
27. S. J. Anderson, S. D. Levin, R. M. Perlmutter, *Nature* **365**, 552 (1993).

28. We thank K. Latinis, E. Peterson, and M. Welsh for critical reading of the manuscript; T. Waldschmidt for antibodies; R. Mulligan for the pPNT plasmid; A. Nagy for the ES cell line; and D. Yablonski and A. Weiss for communicating results before publication. We also thank P. Ruther for technical assistance and animal care and J. Fishbaugh and G. Hess for assistance with flow cytometry. Supported in part by NIH grants GM53256 (G.A.K.) and HL09431 (B.Y.), the Arthritis Foundation (J.L.C.), and the American Heart Association (G.A.K.).

8 April 1998; accepted 29 May 1998

Corelease of Two Fast Neurotransmitters at a Central Synapse

Peter Jonas,* Josef Bischofberger, Jürgen Sandkühler†

It is widely accepted that individual neurons in the central nervous system release only a single fast transmitter. The possibility of corelease of fast neurotransmitters was examined by making paired recordings from synaptically connected neurons in spinal cord slices. Unitary inhibitory postsynaptic currents generated at interneuron-motoneuron synapses consisted of a strychnine-sensitive, glycine receptor–mediated component and a bicuculline-sensitive, γ-aminobutyric acid $(GABA)_A$ receptor–mediated component. These results indicate that spinal interneurons release both glycine and GABA to activate functionally distinct receptors in their postsynaptic target cells. A subset of miniature synaptic currents also showed both components, consistent with corelease from individual synaptic vesicles.

Synaptic transmission in the central nervous system (CNS) is mediated by the release of neurotransmitters into the synaptic cleft and the subsequent activation of postsynaptic receptors. A single neurotransmitter can coactivate multiple ionotropic and metabotropic receptor types (*1*), and a fast neurotransmitter can be coreleased with neuropeptides (*2*). In the spinal cord and brainstem, both glycine and GABA mediate inhibitory synaptic transmission (*3*). It is not known, however, whether glycine and GABA are released from separate or overlapping populations of interneurons. Glycine- and GABA-like immunoreactivity coexist in the somata and boutons of subpopulations of spinal interneurons, and glycine receptor (GlyR) subunit, $GABA_A$ re-

Physiologisches Institut der Universität Freiburg, D-79104 Freiburg, Germany.

*To whom correspondence should be addressed. E-mail: jonasp@ruf.uni-freiburg.de

†On sabbatical leave from II. Physiologisches Institut, D-69120 Heidelberg, Germany.