or SYP immunoprecipitates, and the resulting levels of $p52^{SHC}$ phosphorylation were visualized by anti-phosphotyrosine immunoanalysis (Fig. 4, A and B). Both immunoprecipitated SYP and CTLA-4–associated SYP showed significant phosphatase activity toward $p52^{SHC}$. Thus, we have identified $p52^{SHC}$ as a substrate for the CTLA-4–associated SYP. These findings link SYP, through its ability to dephosphorylate $p52^{SHC}$, as a negative regulator of the RAS pathway.

In the absence of CTLA-4, tyrosine kinase activity and tyrosine phosphorylation levels of $p52^{SHC}$ and CD3ζ are significantly increased in T cells. We show here that tyrosine-phosphorylated CD3ζ recruits $p52^{SHC}$-GRB2 complexes and that MAPK activity is elevated. These findings suggest that the RAS pathway is constitutively activated in *Ctla-4*$^{-/-}$ T cells. SYP appears to be involved in mediating signals for a number of different surface molecules, such as hematopoietic (*17*) and growth factor receptors (*16–18*). Unlike the hematopoietic receptors, growth factor receptors typically contain cytoplasmic tyrosine kinase domains that are autophosphorylated and activated after ligand stimulation (*18*). Activation of the platelet-derived growth factor receptor (PDGFR) has been shown to result in the binding and phosphorylation of SYP, with the subsequent formation of SYP-GRB2 complexes (*18*). Even though in the case of PDGFR, the phosphorylation of SYP correlates with its ability to bind GRB2, we found no evidence of SYP tyrosine phosphorylation in activated mutant or wild-type T cells.

Several reports have shown that association of the phosphatase SHP with receptors such as CD22 in B cells, the erythropoietin receptor, and the natural killer cell inhibitory receptor p58 abrogates signaling from both the B cell and erythropoietin receptors, respectively, and negatively regulates the ability of activated natural killer cells to mediate cytotoxicity (*13*, *14*, *19*). Analogous to the ability of these receptors to recruit SHP in order to abrogate cellular functions, we hypothesize that a function for CTLA-4 is to recruit SYP and that the major role of SYP is to down-regulate T cell activation.

REFERENCES AND NOTES

1. J. M. Green *et al.*, *Immunity* **1**, 501 (1994); T. L. Wallunas *et al.*, *ibid.*, p. 405; M. F. Krummel and J. P. Allison, *J. Exp. Med.* **182**, 459 (1995).
2. J. A. Gross, E. Callas, J. P. Allison, *J. Immunol.* **149**, 380 (1992); R. H. Schwartz, *Cell* **71**, 1065 (1992); P. S. Linsley and J. A. Ledbetter, *Annu. Rev. Immunol.* **11**, 191 (1993); C. H. June, J. A. Bluestone, L. M. Nadler, C. B. Thompson, *Immunol. Today* **15**, 321 (1994); J. P. Allison, *Curr. Opin. Immunol.* **6**, 414 (1994); M. K. Jenkins, *Immunity* **1**, 443 (1994); J.-F. Brunet *et al.*, *Nature* **328**, 267 (1987).
3. P. Waterhouse *et al.*, *Science* **270**, 985 (1995); E. A. Tivol *et al.*, *Immunity* **3**, 541 (1995).
4. M. Okumura and M. L. Thomas, *Curr. Opin. Immunol.* **7**, 312 (1995); J. Bolen, *ibid.*, p. 306; C. E. Rudd, J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, S. F. Schlossman, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5190 (1988); L. E. Samelson, A. F. Philips, E. T. Luong, R. D. Klausner, *ibid.* **87**, 4358 (1990); T. Mustelin, *Immunity* **1**, 351 (1994).
5. A. C. Chan, B. A. Irving, J. D. Fraser, A. Weiss, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9166 (1991); A. C. Chan, M. Iwashina, C. W. Turck, A. Weiss, *Cell* **71**, 649 (1992); R. L. Wange, A.-N.-T. Kong, L. Samelson, *J. Biol. Chem.* **267**, 11685 (1992).
6. K. S. Ravichandran *et al.*, *Science* **262**, 902 (1993); A. E. Nel, S. Gupta, L. Lee, J. A. Ledbetter, S. B. Kanner, *J. Biol. Chem.* **270**, 18428 (1995); M. Iwashima, B. A. Irving, N. S. C. van Oers, A. C. Chan, A. Weiss, *Science* **263**, 1136 (1994); A. Weiss, *Cell* **73**, 209 (1993); ——— and D. R. Littman, *ibid.* **76**, 263 (1994).
7. N. S. van Oers *et al.*, *Mol. Cell. Biol.* **13**, 5771 (1993); N. S. van Oers, N. Killeen, A. Weiss, *Immunity* **1**, 675 (1994).
8. N. Osman, S. C. Lucas, H. Turner, D. Cantrell, *J. Biol. Chem.* **270**, 13981 (1995).
9. L. A. Quilliam, R. Khosravi-Far, S. Y. Huff, C. J. Der, *BioEssays* **17**, 395 (1995); C. J. Marshall, *Cell* **80**, 179 (1995); T. Hunter, *ibid.*, p. 225.
10. L. Buday, S. E. Egan, P. R. Viciana, D. A. Cantrell, J. Downward, *J. Biol. Chem.* **269**, 9019 (1994); M. Sieh, A. Batzer, J. Schlessinger, A. Weiss, *Mol. Cell. Biol.* **14**, 4435 (1994).
11. X. Huang, Y. Li, K. Tanaka, K. G. Moore, J. I. Hayashi, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11618 (1995).
12. C. Pleinman, D. D'Ambrosio, J. Cambier, *Immunol. Today* **15**, 393 (1994).
13. G. M. Doody *et al.*, *Science* **269**, 242 (1995); C.-L. Law, *J. Exp. Med.* **183**, 547 (1996).
14. J. G. Cyster and C. C. Goodnow, *Immunity* **2**, 13 (1995).
15. Lymph node tissue from wild-type C57BL/6 mice was pushed through a fine wire mesh in phosphate-buffered saline to obtain single cells, then suspended in HL-1 medium [2% fetal calf serum, 2 mM Gln], and spread onto plates coated with anti-CD3ε (clone 145-2C11, Pharmingen). After 48 hours, the medium was removed and replaced with 10 ml of HL-1 medium containing interleukin-2. Cells were harvested 2 days later for lysis and immunoprecipitations.
16. G.-S. Feng, C.-C. Hui, T. Pawson, *Science* **259**, 1607 (1993).
17. L. M. Wang, A. Keegan, M. Frankel, W. E. Paul, J. H. Pierce, *Stem Cells* **13**, 360 (1995); T. Yi and J. N. Ihle, *Mol. Cell. Biol.* **13**, 3350 (1993); T. Yi, A. L. F. Mui, G. Krystal, J. N. Ihle, *ibid.*, p. 7577; T. Yi, J. Zhang, O. Miura, J. N. Ihle, *Blood* **85**, 87 (1995).
18. W. Li *et al.*, *Mol. Cell. Biol.* **14**, 509 (1994).
19. M. L. Thomas, *J. Exp. Med.* **181**, 1953 (1995); U. Klingmuller, U. Lorenz, L. C. Cantley, B. G. Neel, H. F. Lodish, *Cell* **80**, 729 (1995); D. N. Burshtyn *et al.*, *Immunity* **4**, 77 (1996).
20. W. Li, C. D. Whaley, A. Mondino, D. L. Mueller, *Science* **271**, 1272 (1996).
21. S.-H. Shen, L. Bastien, B. I. Posner, P. Chretien, *Nature* **352**, 736 (1991).
22. B. Zanke *et al.*, *Eur. J. Immunol.* **22**, 235 (1992); M. Adachi *et al.*, *Biochem. Biophys. Res. Commun.* **186**, 1607 (1992).
23. A. Charest, J. Wagner, S.-H. Shen, M. L. Tremblay, *Biochem. J.* **308**, 425 (1995).
24. L. E. M. Marengère *et al.*, *Nature* **369**, 502 (1994).

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Direct Regulation of ZAP-70 by SHP-1 in T Cell Antigen Receptor Signaling

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The threshold at which antigen triggers lymphocyte activation is set by the enzymes that regulate tyrosine phosphorylation. Upon T cell activation, the protein tyrosine phosphatase SHP-1 was found to bind to the protein tyrosine kinase ZAP-70. This interaction resulted in an increase in SHP-1 phosphatase activity and a decrease in ZAP-70 kinase activity. Expression of a dominant negative mutant of SHP-1 in T cells increased the sensitivity of the antigen receptor. Thus, SHP-1 functions as a negative regulator of the T cell antigen receptor and in setting the threshold of activation.

A dynamic balance between positive and negative regulatory mechanisms is important in setting and maintaining the threshold at which ligands trigger signal transduction. With regard to the T cell antigen receptor (TCR), changes in tyrosine phosphorylation are responsible for initiating signal transduction events. Both SRC family members and ZAP-70 are responsible for the initial changes in tyrosine phosphorylation (*1*). Equally important, but less well understood, are the mechanisms that negatively regulate signal transduction. The SHP-1 protein, a SRC homology 2 (SH2)–containing intracellular protein tyrosine phosphatase (previously termed SHP, SH-PTP1, PTP1C, or HCP) has been implicat-

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ed in the negative regulation of many immunoreceptors (2–4). We sought to determine whether SHP-1 functions to negatively regulate the TCR.

SHP-1 is expressed in thymus and T cells, and in situ hybridization of thymic sections with an antisense SHP-1 complementary RNA (cRNA) indicates higher expression of SHP-1 in the thymic medullary area compared with the cortex (5). To examine the role of SHP-1 in TCR signaling, we transiently transfected the T cell lymphoma cell line Jurkat with either wild-type SHP-1 or a mutated SHP-1 cDNA called SHP-1(C453S) in which the active site cysteine (C) at position 453 was mutated to serine (S) (6). This mutation rendered the protein catalytically inactive. Simultaneously, cells were transfected with a reporter construct of luciferase driven by the interleukin-2 (IL-2) promoter as an indicator of activation. Cells were stimulated by either a monoclonal antibody (mAb) clonotypic for Jurkat TCR (C305), phorbol myristate acetate (PMA), or a combination of both. Cells transfected with catalytically inactive SHP-1(C453S) were more sensitive to stimulation through the TCR in conjunction with PMA (Fig. 1A). The increased IL-2 promoter activity is notable especially given that only a proportion of the cells express the SHP-1(C453S) protein in a transient transfection assay. To examine changes in tyrosine phosphorylation upon stimulation of the TCR, we established Jurkat cell lines that stably express SHP-1(C453S) (6). Expression of the dominant negative SHP-1(C453S) resulted in cells that had increased tyrosine phosphorylation in response to anti-TCR (Fig. 1B). This indicates that expression of catalytically inactive SHP-1 blocks the negative regulation of tyrosine kinases associated with the TCR.

We confirmed these findings by transfecting a T cell hybridoma reactive to an allelic variant of hemoglobin (7) with either SHP-1, SHP-1(C453S), or vector DNA alone (6). When compared with cells overexpressing wild-type SHP-1 or with cells transfected with vector alone, T cell hybridomas expressing the SHP-1(C453S) protein had an increased sensitivity to antigen (Fig. 1C). Cells transfected with either SHP-1 or SHP-1(C453S) cDNAs overexpressed the protein approximately 10-fold (Fig. 1D). The expression of the TCR was unchanged in the cells expressing either SHP-1 or SHP-1(C453S) as determined by flow cytometric analysis (5). The T cell hybridoma transfected with cDNA encoding wild-type SHP-1 did not display a shift in the dose response curve, suggesting that overexpression of wild-type SHP-1 does not affect TCR signaling. Thus, catalytically inactive SHP-1 can interfere with wild-type SHP-1 function and is involved in setting the threshold of antigen receptor signaling by antagonizing positive regulatory mechanisms.

SHP-1 is a cytosolic enzyme regulated by an allosteric mechanism involving the SH2 domains (8–11). Peptides that bind to SHP-1 SH2 domains increase phosphatase activity (3, 10). Negative regulation of cytokine receptors by SHP-1 involves a direct binding of the SH2 domains to the tyrosine-phosphorylated cytoplasmic domain of the receptor (4, 12). Negative regulation of the B cell antigen receptor (membrane immunoglobulin) by SHP-1, at least in part, is mediated by binding to either CD22 or Fcγ receptor type IIB (FcγRIIB) through anchoring, colocalizing, and activating SHP-1 in proximity to the antigen receptor (3, 13).

To determine whether SHP-1 interacts with and dephosphorylates components of the TCR signaling complex, we performed coexpression studies using a vaccinia expression system. HeLa cells were transfected with a cDNA encoding a fusion protein of the extracellular domain and transmembrane portion of vesicular stomatitis virus G protein, and the cytoplasmic domain of the TCR ζ chain (G-ζ), $p59^{fyn}$, and ZAP-70 and either SHP-1 or SHP-1(C453S) (Fig. 2A) (14). Cells expressing SHP-1 had reduced tyrosine phosphorylation of G-ζ and ZAP-70, even in the presence of similar $p59^{fyn}$ kinase activity. These experiments do not eliminate the possibility that SHP-1 may regulate SRC family member kinase activity but indicate that SHP-1 can decrease the tyrosine phosphorylation state of ZAP-70 and G-ζ. This suggests that SHP-1 can negatively regulate TCR signaling. We confirmed this observation by infecting Sf9 insect cells with baculovirus encoding the kinase domain of $p56^{lck}$, ZAP-70, and SHP-1 (14). Expression of SHP-1 decreased ZAP-70 phosphorylation and kinase activity (Fig. 2B).

The coexpression studies in HeLa and Sf9

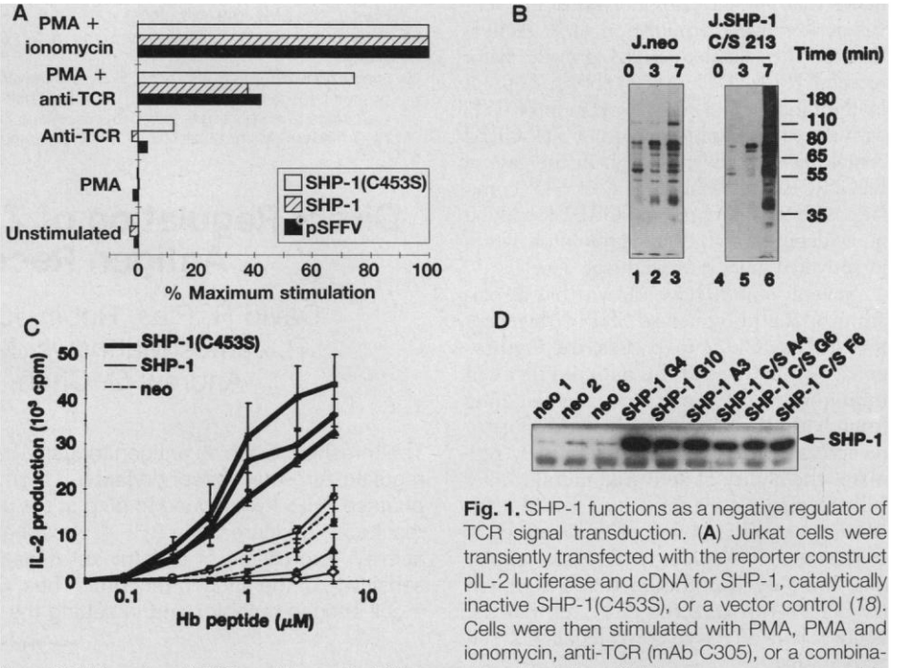

Fig. 1. SHP-1 functions as a negative regulator of TCR signal transduction. (**A**) Jurkat cells were transiently transfected with the reporter construct pIL-2 luciferase and cDNA for SHP-1, catalytically inactive SHP-1(C453S), or a vector control (18). Cells were then stimulated with PMA, PMA and ionomycin, anti-TCR (mAb C305), or a combination of PMA and anti-TCR and harvested 6 hours after stimulation. Cell lysates were measured in duplicate for luciferase activity. Average luciferase activity is presented as percent of maximum stimulation achieved with PMA and ionomycin after subtraction of background. The data shown are representative of six independent experiments. (**B**) A Jurkat clone J.neo transfected with the vector control, lanes 1 to 3 (6), and a Jurkat cell clone with stable expression of SHP-1(C453S), J.SHP-1 C/S 213, lanes 4 to 6, were stimulated with anti-TCR and harvested immediately (time 0) (lanes 1 and 4), 3 min (lanes 2 and 5), and 7 min (lanes 3 and 6) later. Cell lysates from equal cell numbers were separated on an SDS-polyacrylamide gel and immunoblotted with anti-phosphotyrosine (antibody 4G10). Molecular sizes are indicated on the right in kilodaltons. (**C**) The 3L2 T cell hybridoma reactive to mouse hemoglobin(64–76) (Hb) was transfected with either cDNA for SHP-1, catalytically inactive SHP-1(C453S), or vector control (neo) (6). Three individual clones were examined for each transfection and stimulated with increasing concentrations of hemoglobin peptide for 24 hours. Supernatants were measured for IL-2 production with a CTLL proliferation assay. Results shown are representative of five separate experiments. (**D**) Overexpression of SHP-1 and SHP-1(C453S) in the 3L2 T cell hybridoma. SHP-1 protein amounts for the individual clones were determined by immunoblot analysis of cell lysates with an antiserum to the SHP-1 SH2 domain.

cells suggested that SHP-1 and ZAP-70 interact upon T cell activation. To address this question, we stimulated Jurkat cells by either cross-linking the TCR or by treatment with pervanadate, and the ability of SHP-1 and ZAP-70 to interact was measured by coimmunoprecipitation (Fig. 3A) (*15*). No interaction between SHP-1 and ZAP-70 was seen in unstimulated cells. However, activation of T cells by either TCR cross-linking or pervanadate stimulation resulted in the association of SHP-1 and ZAP-70. Immunoblot analysis indicated that ZAP-70 was tyrosine-phosphorylated, identifying ZAP-70 as a tyrosine-phosphorylated protein that coimmunoprecipitates with SHP-1. Thus, after T cell activation SHP-1 and ZAP-70 can interact, which leads to the recruitment and negative regulation of the kinase.

To define the region by which SHP-1 interacts with ZAP-70, we mixed Jurkat T cell lysates from cells stimulated with pervanadate with GST fusion proteins of either the tandem SHP-1 SH2 domains or the SHP-1(C453S) phosphatase domain (Fig. 3B). Both the SHP-1 SH2 domains and the catalytically inactive phosphatase domain bound ZAP-70. We interpret this data to indicate that a tyrosine phosphorylation site within ZAP-70 interacts with SHP-1 SH2 domains. Because catalytically inactive phosphatases can form complexes with their substrates (*16*), it is likely that the catalytically inactive phosphatase domain of SHP-1 forms a complex with ZAP-70 because ZAP-70 is a substrate of the phosphatase. This suggests a possible molecular mechanism by which catalytically inactive SHP-1(C453S) could function as a dominant negative mutant.

SHP-1 in its native state has low catalytic activity, which can be increased by peptides that bind to the SH2 domains (*3, 8, 10*). Although the interaction between SHP-1 and ZAP-70 results in decreased ZAP-70 tyrosine phosphorylation and kinase activity, it is possible that there is a reciprocal relation in that the interaction also results in increased SHP-1 phosphatase activity. We determined whether interaction with ZAP-70 serves to increase phosphatase activity by mixing SHP-1 with a fourfold molar excess of purified ZAP-70 or purified ZAP-70 that had been previously phosphorylated and activated by the $p56^{lck}$ kinase domain (Fig. 4). Interaction between ZAP-70 and SHP-1 increased phosphatase activity (*17*). The phosphatase activity was further increased when ZAP-70 was phosphorylated by $p56^{lck}$. ZAP-70 that had not been incubated with $p56^{lck}$ kinase was also phosphorylated, but to a much lesser extent. It is unlikely that the increase in phosphatase activity is due to phosphorylation of SHP-1 because the assay is performed in the absence of ATP. A CD22 phosphotyrosyl-containing peptide that binds to the NH_2-terminal SHP-1 SH2 domain was required at a 100-fold molar excess to achieve similar activation (*5*). Thus, on a molar basis, ZAP-70 is a more potent stimulator of phosphatase activity than are phosphotyrosyl peptides.

Our data show that the interaction between SHP-1 and ZAP-70 increases phosphatase activity with a concomitant decrease in kinase activity, suggesting that T cell activation is controlled by an autoregulatory loop. The activation of the ZAP-70 tyrosine kinase results in the recruitment of

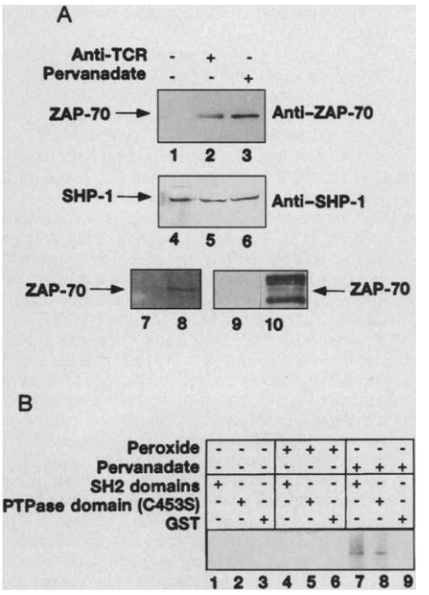

Fig. 3. SHP-1 associates with ZAP-70 upon T cell activation. (**A**) Jurkat cells unstimulated (lanes 1, 4, 7, and 9), stimulated for 2 min with anti-TCR (lanes 2, 5, and 8), or stimulated with pervanadate (lanes 3, 6, and 10) were lysed as described (*15, 20*) and immunoprecipitated with antiserum to SHP-1. Samples were immunoblotted with either anti–ZAP-70 (lanes 1 to 3), anti–SHP-1 (lanes 4 to 6), or anti-pTyr (lanes 7 to 10). (**B**) GST fusion proteins of either the SHP-1 SH2 domains or the SHP-1(C453S) phosphatase domain, or GST alone were added to lysates from untreated (−) (lanes 1 to 3), peroxide-treated (lanes 4 to 6) or pervanadate-treated cells (lanes 7 to 9). Glutathione agarose beads were added, collected, and washed (*15*). The resulting proteins were eluted, resolved on an 8% SDS-polyacrylamide gel, and analyzed for ZAP-70 by immunoblot. PTPase, protein tyrosine phosphatase.

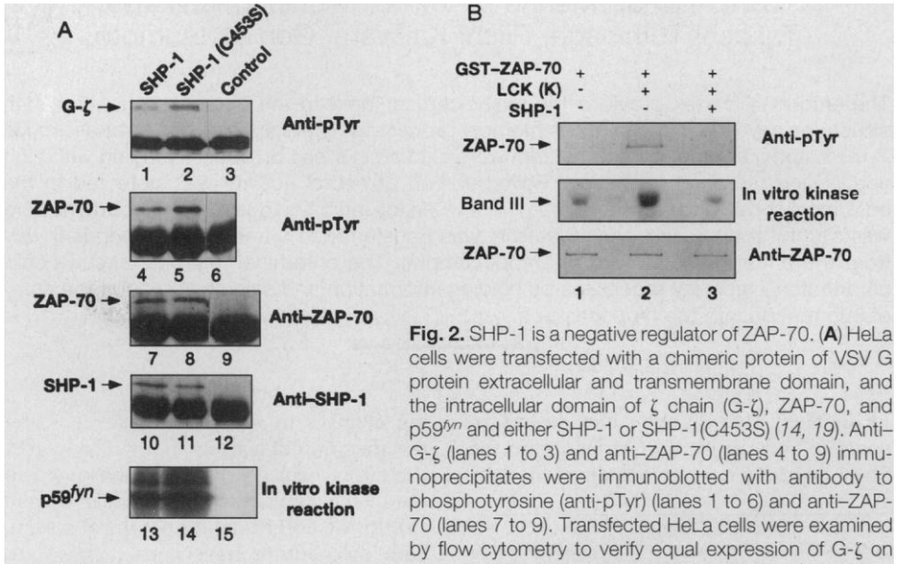

Fig. 2. SHP-1 is a negative regulator of ZAP-70. (**A**) HeLa cells were transfected with a chimeric protein of VSV G protein extracellular and transmembrane domain, and the intracellular domain of ζ chain (G-ζ), ZAP-70, and $p59^{fyn}$ and either SHP-1 or SHP-1(C453S) (*14, 19*). Anti–G-ζ (lanes 1 to 3) and anti–ZAP-70 (lanes 4 to 9) immunoprecipitates were immunoblotted with antibody to phosphotyrosine (anti-pTyr) (lanes 1 to 6) and anti–ZAP-70 (lanes 7 to 9). Transfected HeLa cells were examined by flow cytometry to verify equal expression of G-ζ on cells (*5*). SHP-1 and SHP-1(C453S) expression was determined by SHP-1 immunoprecipitation followed by immunoblot analysis (lanes 10 to 12). $p59^{fyn}$ activity was determined by an in vitro kinase reaction (lanes 13 to 15). Control lanes represent mock-transfected HeLa cells. (**B**) Sf9 insect cells were infected with recombinant baculovirus encoding a fusion protein of glutathione S-transferase–ZAP-70 (GST–ZAP-70), lane 1; GST–ZAP-70 and $p56^{lck}$ kinase domain [LCK (K)], lane 2; or GST–ZAP-70, LCK (K), and SHP-1, lane 3 (*14, 19*). GST–ZAP-70 was isolated on glutathione agarose and subjected to either immunoblot analysis or a kinase reaction. Top panel, anti-pTyr immunoblot; middle panel, in vitro kinase assay with Band III as an exogenous substrate; bottom panel, anti–ZAP-70 immunoblot.

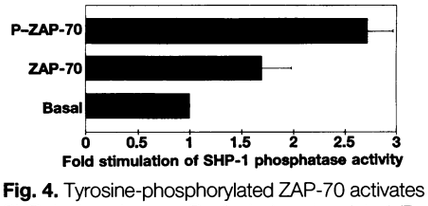

Fig. 4. Tyrosine-phosphorylated ZAP-70 activates SHP-1 phosphatase. Purified phosphorylated (P–ZAP-70), or unphosphorylated ZAP-70, was mixed with SHP-1 in a 4:1 molar ratio. Phosphatase activity was determined with *p*-nitrophenylphosphate as a substrate (*17*). The fold increase in activity is compared with the activity of SHP-1 alone. Preparations of ZAP-70 or phosphorylated ZAP-70 contained negligible phosphatase activity.

SHP-1 that in turn functions to inactivate the kinase and negatively regulate TCR signal transduction. Therefore, the kinase is directly responsible for its own negative regulation.

REFERENCES AND NOTES

1. L. Howe and A. Weiss, *Trends Biochem. Sci.* **20**, 59 (1995).
2. J. G. Cyster and C. C. Goodnow, *Immunity* **2**, 13 (1995).
3. D. D'Ambrosio *et al.*, *Science* **268**, 293 (1995); G. Doody *et al.*, *ibid.* **269**, 242 (1995).
4. U. Klingmüller, U. Lorenz, L. Cantley, B. Neel, H. Lodish, *Cell* **80**, 729 (1995); T. Yi, J. Zhang, O. Miura, J. Ihle, *Blood* **85**, 87 (1995); T. Yi, A. L.-F. Mui, G. Krystal, J. N. Ihle, *Mol. Cell. Biol.* **13**, 7577 (1993).
5. D. R. Plas, R. J. Matthews, G. Roy, A. C. Chan, M. L. Thomas, unpublished data.
6. For transient transfection of Jurkat cells, equal amounts of pIL-2 luciferase and either pSFFV (empty vector), pSFFV-SHP-1, or pSFFV-SHP-1(C453S) were used. After 40 hours, cells were stimulated with PMA (500 ng/ml, Sigma), anti-TCR (antibody C305, 2 μg/ml), or a combination of PMA and anti-TCR, or a combination of PMA and 1 μM ionomycin (Calbiochem). After 6 hours of stimulation, the cells were harvested and lysates were used in a luciferase assay, according to the manufacturer's recommendations (Promega). For stable expression of dominant negative SHP-1(C453S) in Jurkat cells, cells were transfected with either pSFFV or pSFFV-SHP-1(C453S) in which the cDNA was appended at the 3′ end with a sequence encoding an epitope tag derived from c-*myc*. Cells were selected for G418 resistance. The resulting cell lines were cloned by limiting dilution, and individual clones were examined for the expression of SHP-1(C453S) by immunoblotting for the epitope tag. Cell lines were examined for equal expression of the TCR. Individual clones were stimulated with anti-TCR (2 μg/ml) for 0, 3, and 7 min and lysed in ice-cold lysis buffer [1% NP-40, 10 mM tris (pH 8.0), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM leupeptin, 3 mM pepstatin A, 5 mM EDTA, 0.4 mM $NaVO_3$, and 10 mM NaF]. Cell lysates for equal numbers of cells were separated on 8% SDS-polyacrylamide gel and immunoblotted with anti-phosphotyrosine (antibody 4G10, UBI). J.SHP-1 C/S 213 and J.neo are representative clones. 3L2 cells overexpressing SHP-1 or SHP-1(C453S), or vector control cell lines, were generated by transfection of cells with either cytomegalovirus–driven expression vector (BCMG) containing SHP-1 or SHP-1(C453S) cDNA, or vector with no insert. Neomycin-resistant cell lines were obtained, then individual clones were obtained by limiting dilution. Three individual clones were examined for each line. Equivalent expression of CD3 on the resulting clones was determined by flow cytometric analysis. 3L2 hybridoma cells were stimulated for 24 hours with the hemoglobin β peptide(64–76) with CH27 cells used as antigen-presenting cells.
7. B. D. Evavold, S. G. Williams, B. L. Hsu, S, Buus, P. M. Allen, *J. Immunol.* **148**, 3471 (1992).
8. U. Dechert *et al.*, *Eur. J. Biochem.* **231**, 673 (1995); D. Pei, B. G. Neel, C. T. Walsh, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1092 (1993).
9. G. Roy, J. Matthews, T. Woodford-Thomas, M. L. Thomas, *Adv. Protein Phosphatases* **9**, 121 (1995).
10. D. Pei, U. Lorenz, U. Klingmüller, B. G. Neel, C. T. Walsh, *Biochemistry* **33**, 15483 (1994).
11. R. Townley, S.-H. Shen, D. Banville, C. Ramachandran, *ibid.* **32**, 13414 (1993).
12. T. Yi and J. N. Ihle, *Mol. Cell. Biol.* **13**, 3350 (1993).
13. M. Campbell and N. Klinman, *Eur. J. Immunol.* **25**, 1573 (1995).
14. HeLa cells were infected with recombinant vaccinia virus encoding the T7 RNA polymerase. cDNAs in pBluescript (Stratagene) behind the T7 promoter for G-ζ, ZAP-70, SHP-1, SHP-1(C453S), and $p59^{fyn}$ were transfected into HeLa cells. Cells were lysed in 1% NP-40, 10 mM tris (pH 8.0), 150 mM NaCl, aprotinin (50 μg/ml), 2 mM leupeptin, 1 mM PMSF, and 5 mM iodoacetamide, and immunoprecipitation was then performed with specific antiserum. Immunoblotting was performed with 4G10 (anti-phosphotyrosine) and 2f3.2 (anti–ZAP-70) obtained from UBI. For kinase assays, $p59^{fyn}$ immunoprecipitates were incubated at 25°C for 5 min in 10 mM tris (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 10 μCi [γ-P^{32}]adenosine triphosphate (ATP). Plaque-purified and titered baculovirus stocks were used to infect Sf9 cells at 5×10^6 plaque-forming units (pfu) per milliliter. For co-infections, viruses encoding either the GST–ZAP-70 fusion protein (GST–ZAP-70) or $p56^{lck}$ kinase domain were infected at a ratio of 9:1; for infections containing viruses encoding either GST–ZAP-70, $p56^{lck}$ kinase domain, or SHP-1 the ratio was 9:1:2. Cells were also infected with viruses encoding ZAP-70 and SHP-1. Results similar to infection with virus encoding ZAP-70 alone were observed. This is likely because of the requirement of an exogenous kinase to active ZAP-70. Cells were lysed in lysis buffer. ZAP-70 was purified on glutathione agarose (Sigma) according to the manufacturer's recommendations. For kinase assays, ZAP-70 bound to glutathione agarose was incubated at 25°C for 5 min in 10 mM tris (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 10 μCi [γ-P^{32})ATP, with Band III as an exogenous substrate.
15. Jurkat cells were stimulated with anti-TCR (2 μg/ml) for 2 min at 37°C or with 5 mM pervanadate for 10 min at room temperature. Cells were lysed, immunoprecipitated with anti–SHP-1, and analyzed by immunoblot for either SHP-1, ZAP-70, or phosphotyrosine as described. For binding with GST–SHP-1 fusion proteins, Jurkat cells were stimulated with pervanadate as described above or peroxide alone as a control and lysed in 1% brij containing 20 mM tris (pH 7.4), 140 mM NaCl, 5 mM EDTA, 1 mM PMSF, 2 mM leupeptin, aprotinin (20 μg/ml), and 5 mM iodoacetamide. Equal amounts of either GST, GST–SHP-1 SH2 domains (amino acids 1 to 222), or GST–SHP-1 catalytically inactive phosphatase domain (amino acids 237 to 595, C453S) were incubated with lysates from stimulated or unstimulated cells for 1 hour at 4°C and bound to glutathione agarose and Beads were collected by centrifugation and washed three times in brij lysis buffer, and samples were subjected to immunoblot analysis with anti–ZAP-70 (UBI).
16. H. Sun, C. H. Charles, L. F. Lau, N. K. Tonks, *Cell* **75**, 487 (1993); J. B. Bliska, J. C. Clemens, J. E. Dixon, S. Falkow, *J. Exp. Med.* **176**, 1625 (1992).
17. SHP-1 was produced in Sf9 cells and purified by affinity chromatography. A fusion protein of GST–ZAP-70 was produced in Sf9 cells in the presence or absence of $p56^{lck}$ kinase domain as described and purified on glutathione agarose in the presence of 5 mM iodoacetamide. Seven picomoles of ZAP-70 was mixed with 1.75 pmol of SHP-1 in 2 mM *p*-nitrophenylphosphate, 50 mM Hepes (pH 7.0), 5 mM EDTA, and 10 mM dithiothreitol for 1 hour at 37°C, and the amount of *p*-nitrophenylate was determined by measuring absorbance at 405 nm. Phosphatase activity in preparations of ZAP-70 and phosphorylated ZAP-70 was negligible. The fold increase in activity was determined by comparing it with the activity of SHP-1 alone. Background phosphatase activity was subtracted from the calculation.
18. R. J. Matthews, D. B. Borne, E. Flores, M. L. Thomas, *Mol. Cell. Biol.* **12**, 2396 (1992); R. C. Fuhlbrigge, S. M. Fine, E. R. Unanue, D. D. Chaplin, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5649 (1988).
19. E. D. C. McFarland and M. L. Thomas, *J. Biol. Chem.* **270**, 28103 (1995).
20. A. Chan *et al.*, *EMBO J.* **14**, 2499 (1995).

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Entorhinal-Hippocampal Interactions Revealed by Real-Time Imaging

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The entorhinal cortex provides the major cortical input to the hippocampus, and both structures have been implicated in memory processes. The dynamics of neuronal circuits in the entorhinal-hippocampal system were studied in slices by optical imaging with high spatial and temporal resolution. Reverberation of neural activity was detected in the entorhinal cortex and was more prominent when the inhibition due to γ-aminobutyric acid was slightly suppressed. Neural activity was transferred in a frequency-dependent way from the entorhinal cortex to the hippocampus. The entorhinal neuronal circuit could contribute to memory processes by holding information and selectively gating the entry of information into the hippocampus.

A number of mechanisms have been proposed to underlie memory formation in the central nervous system, including long-lasting changes in synaptic efficacy (*1*) and reverberation of activity in a closed loop of excitatory neurons (*2*) as demonstrated in the cerebellonuclear-pontonuclear system (*3*). In the entorhinal-hippocampal system, both mechanisms have been proposed to underlie the significant contribution of this system to learning and memory (*4*). The hippocampus (*5*) is a crucial structure for memory processes, and the closely associated entorhinal cortex (EC) likely executes a specific role as well (*6*). Superficial layers of the EC receive sensory inputs from parts of

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