relate with delayed disease progression and death (*24*).

Collectively, these data indicate that the extent of viremia, measured as HIV-1 RNA, is the best available surrogate marker of HIV-1 disease progression. Several of the rational criteria for demonstrating the adequacy of a surrogate marker as put forward by DeGruttola *et al.* (*25*) appear to have been met: (i) Base-line HIV-1 RNA concentrations are highly predictive of prognosis. (ii) There is a strong time-dependent prognostic relation between HIV-1 RNA and outcome. And (iii) reduced concentrations of HIV-1 RNA, in response to antiretroviral therapy, are predictive of improved prognosis (*24*). Use of HIV-1 RNA as a surrogate marker should help guide future therapeutic research and individual patient management.

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12. For subjects developing AIDS, the samples tested were from study entry, the first follow-up visit (6 months), the most recent visit before AIDS diagnosis, and equally spaced visits in between. For AIDS-free subjects, the samples tested were from study entry, the 6-month follow-up visit, the last available visit, and equally spaced visits in between.
13. Developed by Chiron, the assay measures HIV-1 RNA associated with viral particles that are separated from 1.0-ml plasma samples by centrifugation at 23,500*g* for 1 hour at 4°C. Performance characteristics of the assay have been described elsewhere (*14*). For our study, the interassay coefficient of variation for the positive control samples tested with each batch of experimental samples was 18%. All samples were coded and the assay operators were blinded to clinical outcomes.
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Regulation of T Cell Receptor Signaling by Tyrosine Phosphatase SYP Association with CTLA-4

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The absence of CTLA-4 results in uncontrolled T cell proliferation. The T cell receptor–specific kinases FYN, LCK, and ZAP-70 as well as the RAS pathway were found to be activated in T cells of *Ctla-4*$^{-/-}$ mutant mice. In addition, CTLA-4 specifically associated with the tyrosine phosphatase SYP, an interaction mediated by the SRC homology 2 (SH2) domains of SYP and the phosphotyrosine sequence Tyr-Val-Lys-Met within the CTLA-4 cytoplasmic tail. The CTLA-4–associated SYP had phosphatase activity toward the RAS regulator $p52^{SHC}$. Thus, the RAS pathway and T cell activation through the T cell receptor are regulated by CTLA-4–associated SYP.

Activation through the T cell receptor (TCR) results in increased surface expression of CTLA-4 (*1*). Although CTLA-4 is homologous to the T cell costimulatory molecule CD28 (*2*), CTLA-4–deficient mice have constitutively activated T cells (*3*). Thus, in contrast to CD28, CTLA-4 appears to be a negative regulator of T cell activation (*3*). Initial events in TCR signaling involve the protein tyrosine kinases FYN, LCK, and ZAP-70 (*4*). We now show that FYN, LCK, and ZAP-70, as well as the RAS pathway, are constitutively activated in the T cells of *Ctla-4*$^{-/-}$ mutant mice. This identifies CTLA-4 as a negative regulator of proximal events after activation through the TCR.

We investigated the most proximal effectors in TCR signaling events, the tyrosine kinases FYN, LCK, and ZAP-70, comparing tyrosine kinase activities in primary T cells isolated from lymph nodes of *Ctla-4*$^{-/-}$ mice. The activities of all three tyrosine kinases were increased in homozygous mutant T cells, as shown by elevated autophosphorylation and increased phosphorylation of coimmunoprecipitating proteins (Fig. 1A). ZAP-70 is tyrosine phosphorylated after TCR stimulation (*5*); we also observed constitutive phosphorylation of ZAP-70 in mutant T cells (Fig. 1B). Increased protein phosphorylation in mutant T cells did not reflect elevated expression of tyrosine kinases, as protein immunoblot analyses of immunoprecipitated FYN, LCK, and ZAP-70 showed similar quantities in wild-type and mutant cells (Fig. 1C). Thus, the tyrosine kinases associated with the proximal events in TCR signaling are hyperactive in T cells in the absence of CTLA-4.

In mutant T cells analyzed ex vivo, we observed a drastic increase in tyrosine phosphorylation of 16- and 50-kD proteins and a moderate increase in phosphorylation of 23-, 36-, and 140-kD proteins (Fig. 2A). The 23-, 36-, and 140-kD proteins are targets of tyrosine phosphorylation after TCR activation (*6*). When the antigen receptor is stimulated, the 16-kD TCR subunit CD3ζ and the 50-kD adapter SRC homology and collagen (SHC) are hyperphosphorylated at tyrosine residues (*6*). As previously reported, CD3ζ has a low amount of constitutive phosphorylation (*7*). Together these data raised the possibility that the 16- and 50-kD hyperphosphorylated proteins in mutant cells might be CD3ζ and $p52^{SHC}$,

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respectively. To investigate this, we immunoprecipitated using antibodies to SHC and CD3ζ and then immunoblotted with anti-phosphotyrosine. Immunoanalyses revealed that both $p52^{SHC}$ and CD3ζ were indeed hyperphosphorylated in mutant T cells (Fig. 2, B and C). The anti-SHC immunoblot confirms that equal amounts of protein were used in all immunoprecipitations (Fig. 2D).

Tyrosine phosphorylation of CD3ζ results in the recruitment of the adapter molecules $p52^{SHC}$ and growth factor receptor–bound protein 2 (GRB2), as well as the guanine nucleotide exchange factor Son-of-Sevenless (SOS) (6). Association of the SOS-GRB2-$p52^{SHC}$ complexes with CD3ζ has been suggested as a possible mechanism by which the TCR could activate the RAS signaling pathway (6, 8). Thus, we analyzed mutant T cells to determine if CD3ζ was constitutively associated with $p52^{SHC}$ and GRB2. Analysis of anti-$p52^{SHC}$ immunoprecipitates showed that detectable amounts of CD3ζ bound to $p52^{SHC}$ were present in *Ctla-4*$^{-/-}$ T cells but not in controls (Fig. 2E). Most of the phosphorylated $p52^{SHC}$ in lysates was associated with CD3ζ, as evidenced by the ability of CD3ζ to coimmunoprecipitate with $p52^{SHC}$ at levels similar to that observed in an anti-GRB2 immunoprecipitate (Fig. 2F). Consistent with this result, the anti-CD3ζ immunoprecipitate was also found to be associated with GRB2 at levels approaching that of an anti-$p52^{SHC}$ immune complex (Fig. 2G). Together these data show that CD3ζ can recruit both $p52^{SHC}$ and GRB2 in mutant T cells.

The constitutive formation of CD3ζ-$p52^{SHC}$-GRB2 complexes suggests a mechanism by which the TCR in *Ctla-4*$^{-/-}$ T cells could induce activation of RAS. The RAS signal transduction cascade includes a number of downstream effectors (9). We found that the mitogen-activated protein kinase (MAPK), which is a well-established downstream effector of RAS, is constitutively activated in *Ctla-4*$^{-/-}$ T cells (Fig. 2H). These results suggest that dysregulated T cell activation in *Ctla-4*$^{-/-}$ mice is, at least in part, due to activated RAS-signaling pathways initiated by tyrosine phosphorylation of CD3ζ. Recently, a 36-kD protein was shown to become increasingly phosphorylated on tyrosine after stimulation through the TCR. Tyrosine phosphorylation of the 36-kD protein mediates its association with GRB2 and other SH2 domain–containing effectors (*10*, *11*), raising the possibility that parallel pathways can mediate TCR stimulatory signals.

B cell receptor activation results in the phosphorylation of the accessory molecule CD22, which then binds the SH2-containing tyrosine phosphatase SHP (*12*, *13*). SHP deficiency has in turn been shown to induce hyperactivation of B cell receptor signaling pathways (*14*). As many signaling strategies are conserved between B and T cell antigen receptors, we investigated whether CTLA-4 could also associate with a protein tyrosine phosphatase. Lysates from ex vivo–activated T cells (*15*) were immunoprecipitated with anti–CTLA-4 and assayed for the presence of specific tyrosine phosphatases. We found that the SH2 domain–containing tyrosine phosphatase SYP (*16*) was specifically asso-

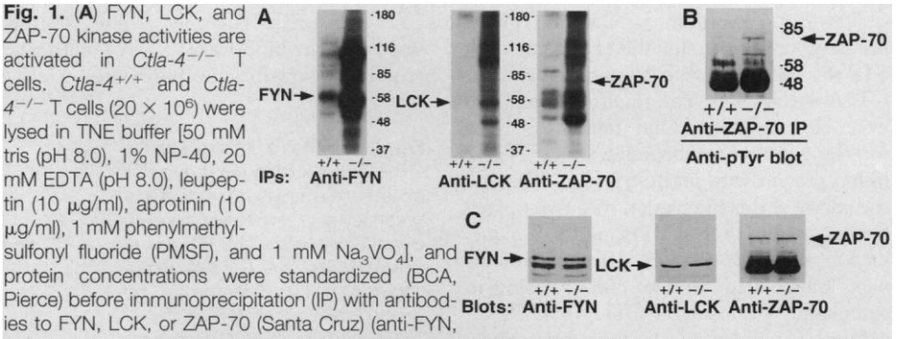

Fig. 1. (**A**) FYN, LCK, and ZAP-70 kinase activities are activated in *Ctla-4*$^{-/-}$ T cells. *Ctla-4*$^{+/+}$ and *Ctla-4*$^{-/-}$ T cells (20×10^6) were lysed in TNE buffer [50 mM tris (pH 8.0), 1% NP-40, 20 mM EDTA (pH 8.0), leupeptin (10 μg/ml), aprotinin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM Na_3VO_4], and protein concentrations were standardized (BCA, Pierce) before immunoprecipitation (IP) with antibodies to FYN, LCK, or ZAP-70 (Santa Cruz) (anti-FYN, -LCK, and –ZAP-70). Immunoprecipitates were washed three times in TNE buffer and twice in kinase buffer [50 mM tris-HCl (pH 7.0), 8 mM $MgCl_2$, 2 mM $MnCl_2$, and 1 mM dithiothreitol]. Kinase reactions were initiated by the addition of 40 μl of kinase buffer and 1 μl of [γ-^{32}P]adenosine triphosphate (ATP) (Amersham), then incubated at 37°C for 10 min. Kinase reactions were stopped by the addition of 40 μl of 2× Laemmli buffer [100 mM tris-HCl (pH 6.8), 20% β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol]. Samples were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The gel was dried and exposed with Kodak-XAR film for 1 hour. (**B**) ZAP-70 is tyrosine-phosphorylated in *Ctla-4*$^{-/-}$ T cells. As in (A), anti–ZAP-70 immunoprecipitates from 20×10^6 *Ctla-4*$^{+/+}$ and *Ctla-4*$^{-/-}$ T cells were separated by SDS-PAGE, transferred, and immunoblotted with antibody to phosphotyrosine (anti-pTyr) (4G10, UBI). Immunoblots were visualized by enhanced chemiluminescence (ECL, Amersham). Molecular sizes in (A) and (B) are indicated in kilodaltons. (**C**) Anti-FYN, anti-LCK, and anti–ZAP-70 immunoprecipitates from 20×10^6 *Ctla-4*$^{+/+}$ and *Ctla-4*$^{-/-}$ T cells were separated and transferred as above, then immunoblotted with either anti-FYN, anti-LCK, or anti–ZAP-70 (Santa Cruz) and visualized by ECL to ensure equal loading of immunoprecipitates in experiments (A) and (B).

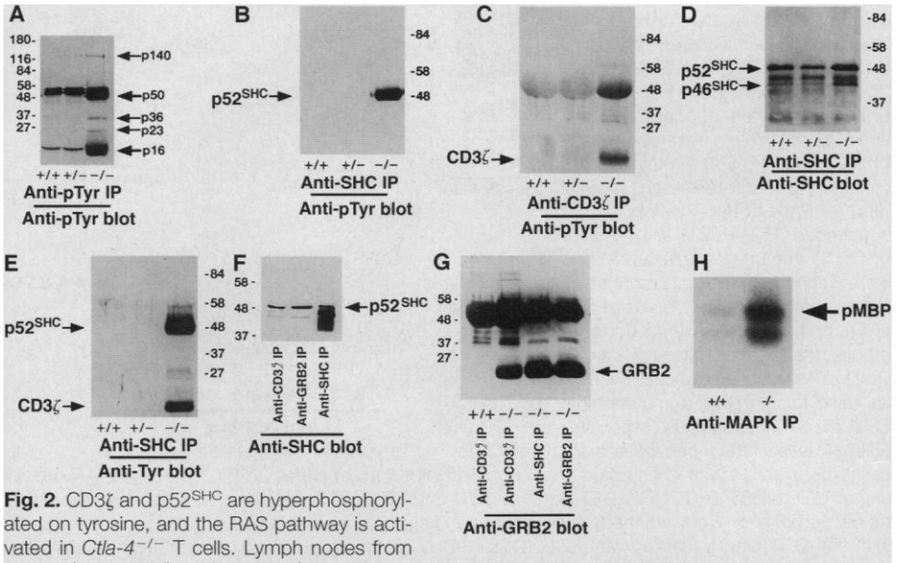

Fig. 2. CD3ζ and $p52^{SHC}$ are hyperphosphorylated on tyrosine, and the RAS pathway is activated in *Ctla-4*$^{-/-}$ T cells. Lymph nodes from *Ctla-4*$^{+/+}$, *Ctla-4*$^{+/-}$, and *Ctla-4*$^{-/-}$ mice were removed and passed through a fine wire mesh to obtain a single-cell suspension. An equivalent number (20×10^6) of *Ctla-4*$^{+/+}$, *Ctla-4*$^{+/-}$, and *Ctla-4*$^{-/-}$ T cells were lysed in TNE buffer. Protein concentrations were precisely standardized (BCA, Pierce), and equal amounts of protein were incubated with either anti-pTyr, anti-SHC, anti-CD3ζ, anti-GRB2 (Signal Transduction Laboratories), or anti-MAPK (Calbiochem), as indicated (IP). Immune complexes from these binding assays were then separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and analyzed by protein immunoblot with either anti-pTyr (**A**, **B**, **C**, and **E**), anti-SHC (**D** and **F**), or anti-GRB2 (**G**). Molecular sizes in (A) through (G) are indicated in kilodaltons. Immunoblots were visualized by ECL. (**H**) The MAPK assay was performed as previously described (*20*). Phosphorylated myelin basic protein (pMBP) was labeled with [γ-^{32}P]ATP (Amersham) and visualized by exposure to Kodak-XAR film for 1 hour.

ciated with CTLA-4 (Fig. 3A). Thus, analogous to CD22 in B cells, CTLA-4 can interact with an SH2 domain–containing tyrosine phosphatase, and this interaction may negatively regulate signaling through the TCR.

We next dissected the physical interactions between SYP and the cytoplasmic tail of CTLA-4. A glutathione S-transferase (GST) fusion protein expressing the SH2 domains of SYP was incubated with immunoprecipitated CTLA-4 (Fig. 3B). The resulting complexes were electrophoresed and immunoanalyzed for the presence of bound SYP GST-SH2 fusion protein with anti-GST (Fig. 3B). The specificity of this interaction was investigated further by separately incubating GST alone, or SYP or GRB2 GST-SH2 fusion proteins with immobilized peptides corresponding to cytoplasmic sequences of CTLA-4. CTLA-4 contains two potential sites for tyrosine phosphorylation, Tyr^{201} [within the sequence Tyr-Val-Lys-Met (YVKM)] and Tyr^{218} [within the sequence Tyr-Phe-Ile-Pro (YFIP)] (*2*). The SYP GST-SH2 fusion protein specifically bound to the CTLA-4 peptide containing the tyrosine-phosphorylated sequence YVKM (Fig. 3C). We further examined CTLA-4–SYP interactions by determining the sensitivity of this association to tyrosine dephosphorylation of the CTLA-4 cytoplasmic tail. Phosphatase treatment of CTLA-4 abrogated the ability of SYP to remain associated to CTLA-4 (Fig. 3D). In addition, peptides containing the phosphorylated YVKM sequence (but not peptides containing the phosphorylated YFIP sequence) abrogate interactions between CTLA-4 and SYP (Fig. 3E). Together, these findings show that the binding of CTLA-4 with SYP requires a tyrosine-phosphorylated YVKM motif in the cytoplasmic tail of CTLA-4.

Our findings to this point suggested that SYP interactions with CTLA-4 may provide a means of down-regulating signals delivered through the TCR. Anti-phosphotyrosine immunoblots had indicated that CD3ζ and $p52^{SHC}$ were hyperphosphorylated in mutant T cells; therefore, we determined if SYP possessed phosphatase activity for $p52^{SHC}$. Equal amounts of tyrosine-phosphorylated $p52^{SHC}$ were added to CTLA-4

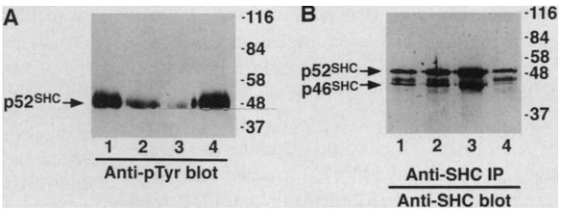

Fig. 4. $p52^{SHC}$ is a substrate for the CTLA-4–associated SYP tyrosine phosphatase activity. (**A**) Activated T lymphocytes (*15*) were lysed and incubated with either anti–CTLA-4 or anti-SYP. The anti–CTLA-4 immunoprecipitate was divided in half and washed with either TNE buffer or TNE containing 2% SDS. Both anti–CTLA-4 and the anti-SYP immunoprecipitates were then washed with phosphatase buffer. Separately, tyrosine-phosphorylated $p52^{SHC}$ was immunoprecipitated from *Ctla-4*$^{-/-}$ T cell lysate, then washed in phosphatase buffer. The SHC immunoprecipitate was separated equally into four portions and either left untreated (lane 1) or added to the anti–CTLA-4 immunoprecipitate (lane 2), the anti-SYP immunoprecipitate (lane 3), or the anti–CTLA-4 immunoprecipitate previously denatured in a final volume of 100 μl (lane 4). Then all complexes were incubated at 37°C for 1 hour. Resulting complexes were washed with TNE buffer, separated, transferred, and immunoanalyzed with anti-pTyr and then analyzed with ECL. (**B**) The anti-SHC immunoblot confirms that an equal amount of immunoprecipitated SHC was present in all samples. Molecular sizes are indicated in kilodaltons.

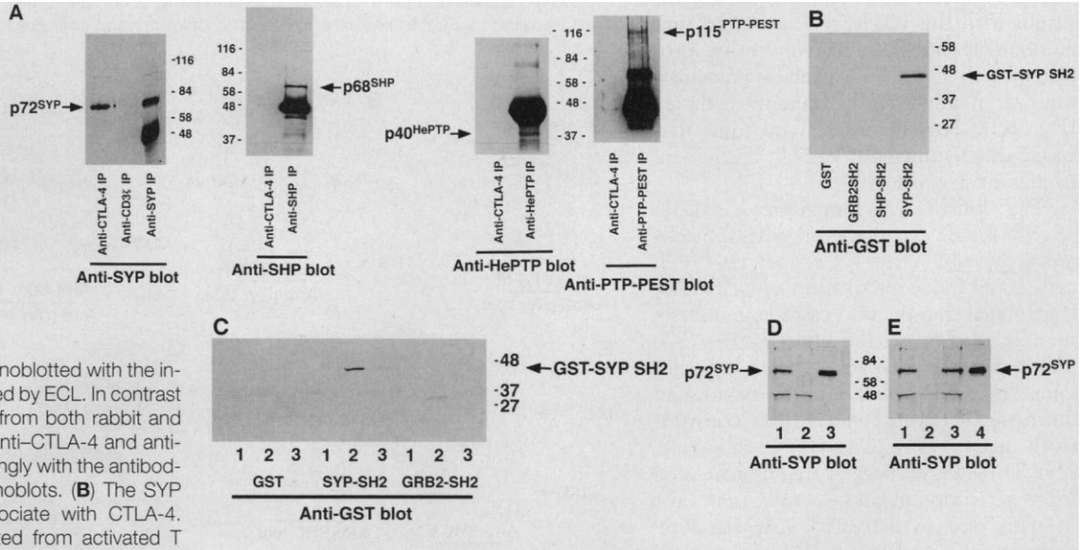

Fig. 3. CTLA-4 associates with the tyrosine phosphatase SYP. (**A**) Activated T lymphocytes (*15*) were lysed in TNE buffer. Cleared lysates were incubated with antibodies to either CTLA-4, SYP (*16*), SHP (*21*) (Signal Transduction Laboratories), hematopoietic-specific protein tyrosine phosphatase (HePTP) (*22*), protein tyrosine phosphatase–containing Pro-Glu-Ser-Thr–rich sequences (PTP-PEST) (*23*), or CD3ζ. Immune complexes were washed, separated, immunoblotted with the indicated antibodies, and visualized by ECL. In contrast to the immunoglobulin G (IgG) from both rabbit and mouse, the IgG from hamster anti–CTLA-4 and anti-CD3ζ does not cross-react strongly with the antibodies used for the protein immunoblots. (**B**) The SYP SH2 domains specifically associate with CTLA-4. CTLA-4 was immunoprecipitated from activated T cells (*15*), washed with TNE buffer, and incubated with 5 μg of either GST alone, GRB2-GST SH2, SHP-GST SH2, or SYP-GST SH2 domains. Immune complexes were washed, separated, transferred, immunoblotted with anti-GST, and visualized with ECL. (**C**) SYP SH2 domains specifically associate with the Y^{201}VKM binding site. Biotinylated peptides based on the cytoplasmic tail of CTLA-4 [1 μM full-length nonphosphorylated wild-type (lanes 1), phosphorylated Y^{201}VKM (lanes 2), or phosphorylated Y^{218}FIP sequences (lanes 3)] were incubated with 5 μg of either GST alone, SYP-GST SH2, or GRB2-GST SH2 fusion proteins. Complexes were washed in TNE buffer, separated, and analyzed as in (B). GST fusion proteins were expressed and purified as previously described (*24*). Phosphorylated peptides were confirmed for the presence of phosphotyrosine with BIACORE (Pharmacia) as previously described (*24*). (**D**) The association between CTLA-4 and SYP is tyrosine phosphatase sensitive. Activated T lymphocytes were lysed in TNE buffer, and CTLA-4 was immunoprecipitated. CTLA-4 immune complexes were washed three times with TNE buffer, then either left untreated (lane 1) or incubated in phosphatase buffer [20 mM Hepes (pH 7.0), 5% glycerol, 0.05% Triton X-100, 2.5 mM $MgCl_2$, aprotinin (10 μg/ml), and leupeptin (10 μg/ml)] at 37°C for 1 hour (lane 2). An anti-SYP immunoprecipitate was included as a positive control (lane 3). Resulting anti–CTLA-4 complexes were electrophoresed and transferred as above, immunoblotted for the presence of SYP, and visualized with ECL. (**E**) CTLA-4 was immunoprecipitated from activated T cell lysates (*15*) as above. Immunoprecipitates were then incubated either without peptide (lane 1) or with 1 μM peptide containing either the phosphorylated sequence Y^{201}VKM (lane 2) or the phosphorylated sequence Y^{218}FIP (lane 3) for 1 hour at 4°C. An anti-SYP immunoprecipitate was included as a positive control (lane 4). Washed immune complexes were separated, transferred, and immunoblotted for the presence of SYP. Molecular sizes are indicated in kilodaltons.

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.

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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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