Reconstitution of T Cell Receptor Signaling in ZAP-70–deficient Cells by Retroviral Transduction of the *ZAP-70* Gene

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Summary

A variant of severe combined immunodeficiency syndrome (SCID) with a selective inability to produce CD8 single positive T cells and a signal transduction defect in peripheral CD4^{+} cells has recently been shown to be the result of mutations in the *ZAP-70* gene. T cell receptor (TCR) signaling requires the association of the ZAP-70 protein tyrosine kinase with the TCR complex. Human T cell leukemia virus type I–transformed CD4^{+} T cell lines were established from ZAP-70–deficient patients and normal controls. ZAP-70 was expressed and appropriately phosphorylated in normal T cell lines after TCR engagement, but was not detected in T cell lines from ZAP-70–deficient patients. To determine whether signaling could be reconstituted, wild-type *ZAP-70* was introduced into deficient cells with a *ZAP-70* retroviral vector. High titer producer clones expressing ZAP-70 were generated in the Gibbon ape leukemia virus packaging line PG13. After transduction, ZAP-70 was detected at levels equivalent to those observed in normal cells, and was appropriately phosphorylated on tyrosine after receptor engagement. The kinase activity of ZAP-70 in the reconstituted cells was also appropriately upregulated by receptor aggregation. Moreover, normal and transduced cells, but not ZAP-70–deficient cells, were able to mobilize calcium after receptor ligation, indicating that proximal TCR signaling was reconstituted. These results indicate that this form of SCID may be corrected by gene therapy.

SCID is a heterogeneous group of genetic disorders characterized by an absence of T and B lymphocyte function. Recently, one form of autosomal recessive SCID has been shown to be caused by defects in the ZAP-70 protein tyrosine kinase (PTK; 1–3). The phenotype of ZAP-70–deficient SCID is distinctive with an absence of peripheral CD8^{+} T cells and normal to high levels of peripheral CD4^{+} T cells that cannot signal through the TCR. ZAP-70 has a relative molecular mass of 70-kD, and is expressed exclusively in thymocytes, T cells, and NK cells (4). It contains two tandemly arranged Src homology 2 (SH2) domains and a carboxy-terminal kinase domain (5).

Engagement of the TCR normally results in activation of intracellular signal transduction pathways culminating in gene expression and cellular proliferation (6). Much evidence indicates that initiation of the signal is mediated through phosphorylation of two tyrosines located within a 17–amino acid motif that is present in all conserved chains

of the TCR, termed the immunoreceptor tyrosine activation motif (ITAM; 7–9). The Src family PTKs Lck and Fyn have been shown to play important roles in the tyrosine phosphorylation of these ITAMs (10–12). ZAP-70 can then associate with the phosphorylated ITAMs of the CD3-ζ and CD3-ε chains of the TCR through its SH2 domains (5, 13, 14). ZAP-70 is itself phosphorylated upon this interaction, in a process that is dependent on Src family PTKs (5, 14–16). The subsequent steps in the TCR signaling pathway that result in T cell proliferation and cytokine release are not well defined. However, the observation that there is an almost complete abrogation of TCR signal transduction in CD4^{+} cells in ZAP-70–deficient patients (1–3) indicates a critical role for ZAP-70 in this process.

Interestingly, there is a second member of this PTK family, termed Syk, that is structurally homologous to ZAP-70 and shares 57% sequence identity (5, 17). Syk is expressed in a wide variety of cells including B cells, mast cells, gran-

ulocytes, platelets, and thymocytes, but its level in peripheral T cells is significantly lower (<10% of the level in B cells; 4). Additionally, although Syk is known to be important for signaling in B cells, mast cells, and granulocytes, its role in T cell signaling is not clear (18–25). We have recently shown that high levels of endogenous Syk may partially compensate for the absence of ZAP-70 in thymocytes from a ZAP-70–deficient patient, resulting in signaling through the TCR (26). In contrast with thymocyte signaling, however, there is a lack of signaling, as assessed by protein tyrosine phosphorylation and mobilization of $[Ca^{2+}]_i$, in the peripheral $CD4^+$ T cells from this patient (26). This observation again underscores the importance of ZAP-70 in the signal transduction pathway in peripheral T cells.

In the present work, we demonstrate successful reconstitution of TCR–mediated signaling in a $CD4^+$ peripheral T cell line from this ZAP-70–deficient patient. The cell lines have previously been shown to have defective TCR–mediated signaling analogous to that seen in primary cells from the patient (26). Cells were reconstituted by retroviral-mediated transduction with the wild-type *ZAP-70* gene. After transduction of the patient's cell line, TCR signaling was normalized as measured by ZAP-70 phosphorylation, ZAP-70 in vitro kinase activity, and reconstitution of calcium mobilization.

Materials and Methods

Cell Lines. The patient with ZAP-70 deficiency has been reported previously (26). Briefly, HTLV-I transformation of peripheral blood T cells from normal adult donors and the ZAP-70–deficient patient (AB) were performed as described (27). The normal and AB HTLV-I lines were maintained in RPMI 1640 with 10% FCS, IL-2 (20 U/ml; gift from Carolyn Paradise, Chiron Corp., Emeryville, CA), 2 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The phenotype of control and ZAP-70–deficient T cell lines was similar; each expressed TCR-α/β as well as CD4. Additionally, all cell lines were IL-2 dependent. The human T leukemia cell line Jurkat was maintained in RPMI 1640 with 10% FCS.

Construction of the G1ZAPSvNa Vector and Packaging Line. The normal *ZAP-70* cDNA (5) was excised from Bluescript KS(+) as an EcoRI fragment, blunted with Klenow DNA polymerase, and cloned into the SnaBI site of the pG1XSvNa vector plasmid, a Moloney murine leukemia virus–based retroviral vector (28; Genetic Therapy, Inc., Gaithersburg, MD).

To generate a high titer packaging line, the resultant pGZAPSvNa plasmid was transfected into the gp+env 86 ecotropic packaging line using DOTAP reagent (Boehringer Mannheim, Indianapolis IN) (29). Supernatants from the ecotropic line were then used to infect PG13 amphotropic packaging cells (30), and a single clone with a titer of 4 × 10⁵/ml was used for transductions.

Retroviral Transductions. pG1ZAP70SvNa supernatants were added at a 1:1 dilution to AB cells in logarithmic growth phase (10⁵ cells/ml) in the presence of IL-2 (20 U/ml) and 3 μg/ml protamine. Fresh viral supernatants were added every 24 h for 3 successive days. The AB cells were then diluted to ~5 × 10⁴ cells/ml, and 1-ml aliquots were seeded into the wells of a 24-well plate and selected in 400 μg/ml of G418 (geneticin; GIBCO-BRL, Gaithersburg, MD). In three different transductions, approximately four to eight wells from each plate grew after 3–6 weeks of G418 selection. Control cells were transduced with the LN vector, which contains only the neomycin phosphotransferase gene (28).

Immunoprecipitations and Kinase Assays. Cells were starved overnight in RPMI 1640 with 1% FCS (in the absence of IL-2). After washing twice in HBSS, cells were resuspended at 2 × 10⁷ cells/ml, incubated with 100 μl of a biotinylated anti-CD3 antibody (Leu4 mAb; Becton Dickinson & Co., Mountain View, CA) for 15 min on ice, and then cross-linked with streptavidin (30 μg/ml, Calbiochem/Novabiochem Corp., La Jolla, CA) for 2 min at 37°C. Cells were lysed in an NP-40 lysis buffer (31), and postnuclear supernatants were immunoprecipitated with a rabbit polyclonal antibody to ZAP-70, 1222 (5), followed by collection on protein A/G agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunoprecipitates were separated on 7.5% SDS-PAGE gels and transferred electrophoretically to Hybond membranes (Amersham, Arlington Heights, IL).

Membranes were blotted with a mixture of the 4G10 (Upstate Biotechnology Inc., Lake Placid, NY) and pY72 antiphosphotyrosine mAbs (gift from Bart Sefton, Salk Institute, La Jolla, CA) as previously described (31). Blots probed with a ZAP-70–specific mAb, 2F3.2 (14), were blocked in TBS (150 mM NaCl, 20 mM Tris, pH 7.5) containing 5% BSA and 0.1% Tween 20. Immunocomplexes were visualized using the enhanced chemiluminescence detection system (Amersham).

In vitro kinase assays were performed essentially as reported (32). Immunoprecipitates were washed in lysis buffer followed by 150 mM NaCl and 50 mM Tris HCl, pH 7.5. Precipitates were then incubated in 25 μl kinase buffer (20 mM Pipes, pH 7.0, 10 mM MnCl₂, and 5 μCi [γ³²P]ATP [3,000 Ci/mM]) for 10 min at 30°C, washed once, eluted by boiling in 2× SDS sample buffer, resolved on SDS-PAGE, and visualized by autoradiography.

Measurement of Cytoplasmic Calcium Concentration. T cell lines were starved overnight in RPMI 1640 with 10% FCS (in the absence of IL-2), and were loaded with Indo-1 (Molecular Probes, Inc., Eugene, OR) in the same medium at 20°C for 45 min. Cells were washed and resuspended at ~10⁶ cells/ml in HBSS containing 1% BSA. Cells were stimulated with biotinylated anti-Leu4 mAb followed by streptavidin at 37°C in a constantly stirred acrylic cuvette. Fluorescence measurements to determine $[Ca^{2+}]_i$ were performed with a spectrofluorimeter (Photon Technologies, South Brunswick, NJ) at an excitation wavelength of 350 nm (4-nm bandwidth), and with dual simultaneous monitoring of emission at 405 and 485 nm (10-nm bandwidth). The ratio of emission at 405/485 nm was measured at a rate of 2 Hz and is presented as a function of time (s).

Results

Lack of ZAP-70 Expression in a Peripheral $CD4^+$ Cell Line Derived from Patient AB. We have previously shown that patient AB, the product of a consanguineous union, has ZAP-70–deficient SCID (26). This syndrome was diagnosed from the patient's peripheral blood T cell phenotype (adequate $CD4^+$ count with a marked paucity of $CD8^+$ T cells), the failure of his PBMCs to mediate increases in $[Ca^{2+}]_i$, and an absence of detectable ZAP-70 protein. Additionally, no ZAP-70–specific RNA could be detected in thymocytes from patient AB. To study signaling in peripheral $CD4^+$ cells from this patient, an HTLV-I–transformed polyclonal T cell line was derived. HTLV-I–transformed $CD4^+$ T cell lines from normal individuals were used as controls. Like $CD4^+$ HTLV-I lines derived from normal individuals, the patient's HTLV-I T cell line expressed CD4, the Lck and Fyn Src family PTKs, the TCR-ζ chain, and PLC-γ1 (data not shown). All HTLV-I

G1ZAPSvNa

| 5' LTR | Ψ + | ZAP-70 | Sv | neo | 3' LTR |

|← 1 Kb →|
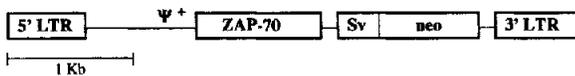
Figure 1. Map of the G1ZAPSvNa vector. Shown are positions of the 5' long terminal repeat (*5' LTR*), *ZAP-70* cDNA, SV-40 promoter/enhancer, neomycin phosphotransferase (*neo*) gene, and 3' long terminal repeat (*3' LTR*).

lines required the presence of exogenous IL-2 (20 U/ml) in the culture medium to sustain growth. In contrast with control HTLV-I lines (*Normal*), however, ZAP-70 expression was not detected in the AB HTLV-I line (*AB*; see Fig. 2 *B*). These data indicated that the AB HTLV-I line could be used as a tool to assess whether the transduction of wild-type ZAP-70 would result in the reconstitution of TCR-mediated signaling.

ZAP-70 Is Expressed and Tyrosine Phosphorylated after Transduction with G1ZAPSvNa. To determine whether wild-type ZAP-70 would restore TCR function in AB T cells, cells were transduced with a retroviral vector G1ZAPSvNa, into which the entire *ZAP-70* cDNA was cloned (Fig. 1). Pools of AB cells from three different transduction experiments were selected in G418 for 3–6 wk to obtain cells containing G1ZAPSvNa (AB/ZAP-70 cells). In these transduced T cell pools, ZAP-70 was expressed at levels ranging from 0.2 to 2.0-fold of the levels observed in control T cell lines (data not shown). In Fig. 2 *B*, ZAP-70 expression in an AB/ZAP-70 T cell pool is equivalent to that observed in a normal T cell line (*Normal*). This indicates that retroviral transduction of ZAP-70 into deficient cells can result in approximately normal levels of ZAP-70 expression. The level of ZAP-70 expressed in six individual pools of AB/ZAP-70–transduced cells did not change after up to 8 mo of continuous culture in the absence of G418, as monitored by immunoblot analysis (data not shown). This result demonstrates that ZAP-70 can be stably expressed in cultured T cells after transduction with the G1ZAPSvNa retroviral vector.

Upon TCR stimulation, ZAP-70 normally associates with phosphorylated ITAMs that are present in the CD3-ε or -ζ chains, and is itself phosphorylated (5, 13–16). Therefore, we assessed whether ZAP-70 would be tyrosine phosphorylated in AB/ZAP-70–reconstituted cells after receptor engagement. Cell lines were rested overnight in the absence of exogenous IL-2 before stimulation of the TCR with a biotinylated anti-CD3 mAb (Leu4) followed by cross-linking with streptavidin. In a normal as well as the reconstituted cell line (AB/ZAP-70), the 70-kD ZAP-70 protein was tyrosine phosphorylated upon TCR aggregation. It is interesting to note that several molecular mass species of >70 kD were also tyrosine phosphorylated, albeit at lower levels, in ZAP-70 immunoprecipitates from α-CD3–stimulated normal and reconstituted cell lines. Because the phosphotyrosine-reactive bands in the AB/ZAP-70–transduced cells were of slightly lower intensity than those observed in the normal T cells, these higher molecular weight species were not evident in the AB/ZAP-70 lane shown in Fig. 2 *A*. On longer ECL exposures, however, the identical pattern of phosphotyrosine-reactive bands
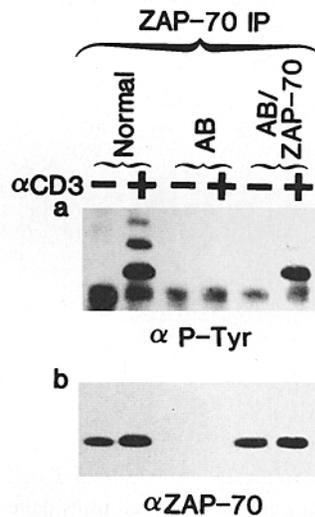
Figure 2. Retroviral-mediated transduction of the wild-type ZAP-70 gene results in ZAP-70 expression and its phosphorylation upon CD3 aggregation. (*A*) ZAP-70 was immunoprecipitated using a polyclonal rabbit antibody from a normal PBL line (*Normal*), a ZAP-70–deficient PBL line (*AB*), and a ZAP-70–deficient PBL line reconstituted by retroviral-mediated transduction with a wild-type ZAP-70 gene (*AB/ZAP-70*). 10⁷ cells were either unstimulated (−) or stimulated (+) with a biotinylated anti-CD3 mAb followed by streptavidin. Immunoprecipitated lysates were fractionated on a polyacrylamide gel, analyzed by immunoblotting with the 4G10 monoclonal antiphosphotyrosine antibody, and developed with enhanced chemiluminescence to assess the phosphorylation status of ZAP-70. (*B*) The blot was stripped and reprobed with a ZAP-70–specific mAb to assess the level of immunoprecipitated ZAP-70.

was also observed in the AB/ZAP-70 cells (data not shown). This result was reproducible in six tested pools of AB/ZAP-70–transduced cells, with levels of ZAP-70 ranging from 0.2- to 2.0-fold of those observed in control T cell lines. No tyrosine-phosphorylated ZAP-70 would be expected, or was observed, in the nontransduced AB cells or in AB cells transduced with the LN vector, since the ZAP-70 protein is not expressed in these cells (Fig. 2 and data not shown). Thus, one of the proximal events of TCR signaling, the tyrosine phosphorylation of ZAP-70 itself, was reconstituted in AB/ZAP-70 cells.

In Vitro Kinase Activity of ZAP-70 Is Activated by TCR Stimulation in AB/ZAP-70 Cells. To investigate whether ZAP-70 is activated as a result of CD3 clustering in the reconstituted ZAP-70–deficient HTLV-I cell line (AB/ZAP-70), an in vitro kinase assay was performed. After CD3 stimulation, immunoprecipitation with a ZAP-70–specific antibody was performed in the Jurkat T cell leukemia clone, normal and AB HTLV-I T cell lines, as well as in two different pools of AB-transduced cells (AB/ZAP-70ₐ and AB/ZAP-70ᵦ). Immunoprecipitates were incubated in the presence of [γ³²P]ATP. ZAP-70 was autophosphorylated in Jurkat T cells, as well as in the normal and reconstituted AB/ZAP-70 cell lines, but not in the AB cell line (Fig. 3). This kinase activity increased upon receptor stimulation (Fig. 3). Within each cell line tested, the level of ZAP-70 protein immunoprecipitated before and after CD3 cross-linking was the same as that assessed by immunoblotting with an anti–ZAP-70 mAb. The level of ZAP-70 in the AB/ZAP-70ᵦ pool (equivalent to that in a normal HTLV-I T cell line), however, was approximately fivefold higher than in the AB/ZAP-70ₐ pool (data not shown), accounting for the lower ZAP-70 kinase activity observed in AB/ZAP-70ₐ, as compared with the normal and AB/ZAP-70ᵦ lines (Fig. 3). Interestingly, a protein with a molecular mass of ~140 kD, which coimmunoprecipitated only in the
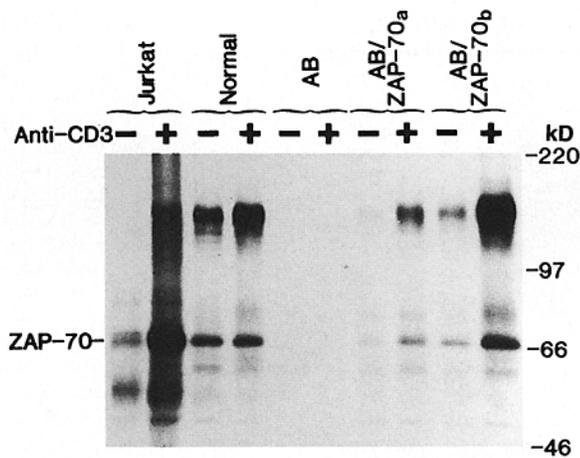
Figure 3. In vitro kinase activity of ZAP-70 is stimulated by TCR aggregation in ZAP-70–reconstituted cells. Jurkat T cells, as well as PBL lines from a normal individual (*Normal*), patient AB, and two different pools of ZAP-70–transduced cells (AB/ZAP-70a and AB-ZAP-70b), were assessed for autophosphorylation. After TCR aggregation (+), lysates from 10⁷ cells were immunoprecipitated with a polyclonal ZAP-70–specific antibody, and an in vitro kinase assay was performed. The amount of ZAP-70 immunoprecipitated before and after stimulation was identical, as assessed by α-ZAP-70 immunoblotting (not shown).

presence of ZAP-70, was also phosphorylated after TCR stimulation. Although the identity of this second protein is not known, its phosphorylation also increased upon TCR ligation in both normal and reconstituted cell lines (Fig. 3). We are presently analyzing whether the 140-kD protein is expressed as a consequence of HTLV-I transformation, and furthermore, whether it can be coimmunoprecipitated with antibodies directed against different ZAP-70 epitopes.

Calcium Mobilization after TCR Stimulation Is Dependent on ZAP-70 Expression. The increases observed in cytosolic calcium concentrations [Ca²⁺]ᵢ after TCR aggregation is absent in ZAP-70–deficient T cells (1–3). Calcium flux is one of the proximal events induced upon TCR stimulation (10–12, 33–35). We therefore assessed whether changes in [Ca²⁺]ᵢ would be observed in reconstituted AB/ZAP-70 cells. For calcium measurements, cells were loaded with the calcium-sensitive dye Indo-1 and stimulated with a biotinylated CD3 mAb followed by streptavidin. Changes in [Ca²⁺]ᵢ were then monitored by fluorimetry. In contrast to AB cells, in which little or no [Ca²⁺]ᵢ flux was noted, normal cells and three different pools of AB/ZAP-70–reconstituted cells (including AB/ZAP-70a and AB/ZAP-70b) had a sustained increase in [Ca²⁺]ᵢ upon receptor cross-linking (Fig. 4 and data not shown). Equivalent loading of all cell lines with Indo-1 was affirmed in all experiments by demonstrating calcium flux after addition of calcium ionophore (ionomycin; data not shown).

Discussion

We have shown that retroviral-mediated transduction of the wild-type *ZAP-70* cDNA into a CD4⁺ HTLV-I–transformed T cell line from a ZAP-70–deficient patient results
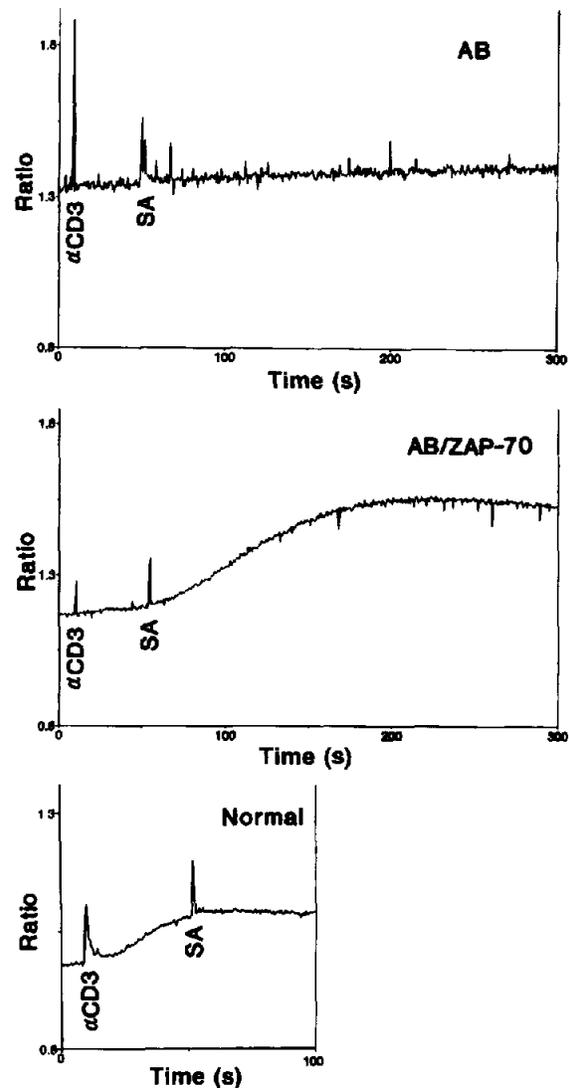
Figure 4. ZAP-70 is required for calcium mobilization after TCR stimulation. Cells from a ZAP-70–deficient PBL line (*AB*), a ZAP-70–reconstituted line (*AB/ZAP-70a*), and a normal PBL line (*Normal*) were loaded with the calcium-sensitive dye Indo-1 and analyzed for increases in [Ca²⁺]ᵢ. Cells were stimulated with a biotinylated α-CD3 antibody (Leu4-B) followed by streptavidin (*SA*), as indicated. Changes in [Ca²⁺]ᵢ were monitored by fluorimetry as a measure of the ratio of emission at 405/485 nm (*ordinate axis*) using a Photon Technologies spectrofluorimeter for the indicated time period (s).

in reconstitution of the proximal TCR signaling pathway, as assessed by the phosphorylation and activation of ZAP-70 itself and by the mobilization of cytosolic calcium. This phenotypic correction was observed in six different pools of T cells transduced with G1ZAPSvNa. Since the level of ZAP-70 in each transduced T cell pool did not vary during >8 mo of culture in the absence of G418 selection, this retroviral-based vector can be used to achieve stable ZAP-70 expression. Upon similar transduction of G1ZAPSvNa into normal HTLV-I–transformed T cells, overexpression of ZAP-70 could not be achieved, suggesting that high levels of ZAP-70 were counterselected in cultured T cells (data not shown). Further analyses in animal models will be nec-

essary to determine whether T cell polyclonality and diversity of the T cell repertoire is maintained after transduction with the G1ZAPSvNa retroviral vector.

Multiple cellular PTKs interact with the TCR and contribute to the signal transduction pathway, including the Src family PTKs Lck and Fyn, as well as the ZAP-70 and related Syk PTKs (10–12, 35–38). Increased levels of Syk were observed in both the HTLV-I–transformed thymocyte and peripheral T cell lines derived from the ZAP-70–deficient patient described in this report (reference 26, and Bacon, K., S. Smith, T. Jahn, T. Kadlecek, A. Weiss, K. Weinberg, and N. Taylor, unpublished observations). Syk appears to compensate for ZAP-70 only under specific cellular conditions; TCR-stimulated calcium flux was observed in the patient's IL-2–dependent thymocyte cell line (26), but not in the IL-2–dependent peripheral T cell line derived from the same patient (Fig. 4). Nevertheless, upon transition of the patient's peripheral T cell line to IL-2 independence with concurrent activation of the JAK/STAT kinase pathway, as described previously (39–41), Syk was sufficient to mediate a calcium response (Bacon, K., and N. Taylor, unpublished observations). Work is in progress to ad-

dress the relative contributions of these two PTKs to TCR signaling in ZAP-70–reconstituted thymocytes and T cells.

Our experiments focused on the role of ZAP-70 in TCR signaling, but the phenotypes of ZAP-70–deficient patients and mice indicate that ZAP-70 also plays an important role in T cell development (1–3, 42). ZAP-70–deficient patients have normal or increased numbers of CD4⁺ cells with a paucity of CD8⁺ cells, whereas mice with a targeted deletion of the *ZAP-70* gene express neither CD8 nor CD4 single positive T cells (42). Introduction of ZAP-70 into hematopoietic stem cells isolated from the ZAP-70–deficient mouse may provide insights into the biological effects of exogenous ZAP-70 expression. It will be important to determine the bases of the differences between ZAP-70–deficient patients and mice, however, since gene therapy protocols for ZAP-70–deficient SCID patients will have to target a progenitor of T lymphocytes that is able to achieve correction of T cell ontogeny. The present experiments demonstrating correction of signal transduction through the TCR in a ZAP-70–deficient T cell line lay the foundation for these further preclinical investigations.

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