Absence of ZAP-70 Prevents Signaling through the Antigen Receptor on Peripheral Blood T Cells but not on Thymocytes

By Erwin W. Gelfand,* Kenneth Weinberg,‡ Bruce D. Mazer,*
Theresa A. Kadlecek,§ and Arthur Weiss§

*From the *Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Diseases, Denver, Colorado 80206; ‡Division of Research Immunology, Children's Hospital, Los Angeles, California 90027; §Howard Hughes Medical Institute, Department of Medicine and of Microbiology and Immunology, University of California, San Francisco, California 94143*

Summary

Recently, a severe combined immunodeficiency syndrome with a deficiency of $CD8^+$ peripheral T cells and a TCR signal transduction defect in peripheral $CD4^+$ T cells was associated with mutations in ZAP-70. Since TCR signaling is required in developmental decisions resulting in mature CD4 (and CD8) T cells, the presence of peripheral $CD4^+$ T cells expressing TCRs incapable of signaling in these patients is paradoxical. Here, we show that the TCRs on thymocytes, but not peripheral T cells, from a ZAP-70–deficient patient are capable of signaling. Moreover, the TCR on a thymocyte line derived from this patient can signal, and the homologous kinase Syk is present at high levels and is tyrosine phosphorylated after TCR stimulation. Thus, Syk may compensate for the loss of ZAP-70 and account for the thymic selection of at least a subset of T cells ($CD4^+$) in ZAP-70–deficient patients.

The severe combined immunodeficiency syndrome (SCID)[1] comprises a group of heterogeneous disorders affecting the functional activity of both T and B lymphocytes (1). Phenotypically, infants with SCID can have either a marked deficiency in circulating lymphocytes or normal numbers of T and B cells whose function is abnormal. T cell immunity abnormalities in SCID have been associated with an absence of T cell differentiation (2), failure to produce IL-2 (3), and mutations in the genes encoding the γ chain of the IL-2 receptor (4, 5) or the CD3 γ subunit of the TCR (6). Recent studies have also described defects in the ZAP-70 protein tyrosine kinase (PTK), which is involved in signal transduction through the TCR (7–9).

Stimulation of TCRs on T lymphocytes leads to the tyrosine phosphorylation of specific intracellular substrates and the subsequent rapid increase in cytoplasmic free Ca^{2+} levels ($[Ca^{2+}]_i$) (10–14). Since the component chains of the TCR do not contain an intrinsic kinase domain, signal transduction must occur via association of these receptors with intracellular protein kinases (PTKs). Two PTK families have been shown to interact with the TCR. Members of the Src family of PTKs, Fyn and Lck, have been implicated in T lymphocyte signal transduction and in T cell differentiation (13, 14). Fyn has been coimmunoprecipitated with the TCR (15) and inactivation of the *fyn* gene impairs signal transduction in mature thymocytes (16, 17). In a T cell line (18) and cytolytic T cell clone (19), Lck has been shown to be required for the induction of tyrosine phosphoproteins, including the TCR CD3 and ζ chains. Moreover, Lck-deficient mice exhibit a profound failure of early T cell development with few thymocytes and circulating T cells that exhibit an impaired response to TCR stimuli (20).

A second family of PTKs, consisting of ZAP-70 and Syk, have also been implicated in TCR-mediated signaling. In T cell lines, ZAP-70 and Syk are recruited to the phosphorylated CD3 and ζ subunits, where they are in turn tyrosine phosphorylated after TCR stimulation (21, 22). The failure of the recruitment of ZAP-70 to the TCR in an Lck-deficient T cell line suggests that it plays a role downstream of Lck (23). It was hypothesized, therefore, that the TCR may interact with members of these two families of PTKs sequentially (24). In this model, an Src family member would first phosphorylate tyrosine residues contained in the ζ and CD3 chains. Only then would ZAP-70 and/or Syk be recruited to the receptor complex via an interaction involving both of their SH2 domains with the phosphorylated receptor chains. Once recruited to the receptor,

[1]*Abbreviations used in this paper:* $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} levels; HTLV, human T lymphotropic virus; PLC, phospholipase C; PTK, protein tryosine kinase; SCID, severe combined immunodeficiency syndrome.

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ZAP-70 and/or Syk can interact with Src family PTKs, resulting in a synergistic increase in PTK activity.

ZAP-70 and Syk are structurally homologous, having two tandemly arranged SH2 domains and a COOH-terminal kinase domain (25, 26). ZAP-70 protein is expressed in T cells and NK cells (26). It is also expressed in thymocytes at levels comparable to those found in peripheral $CD4^+$ and $CD8^+$ T cells (22). A role for ZAP-70 has been established in T cells (see below). Syk has been most clearly implicated in B cell mIg, as well as in mast cell $Fc_\varepsilon RI$ signal transduction (27–30). After mIg or $Fc_\varepsilon RI$ stimulation, Syk associates with the tyrosine-phosphorylated, non–ligand-binding chains of both of these receptors. Genetic evidence for the requisite function of Syk in mIg signal transduction function has been obtained in a chicken B cell line (30). The function of Syk in T cells is not as clear, however. Syk protein is present at higher levels in thymocytes than peripheral T cells, but the level of Syk in peripheral T cells is only ~1/12–1/15 of the level in B cells (22). In a T cell line expressing relatively high levels of Syk, its association with the TCR and its tyrosine phosphorylation after receptor stimulation has been observed (31). Although some data suggest that Syk can interact with the TCR independently of Lck involvement and TCR engagement (31), this is not consistent with our published and more recent observations (22) (Chu, D., N. van Oers, and A. Weiss, unpublished data).

Three recent studies have provided evidence that the absence of ZAP-70 protein, which results from inherited mutations, leads to a form of SCID (7–9). This syndrome is characterized by the absence of peripheral $CD8^+$ T cells and a failure of peripheral $CD4^+$ T cells to signal normally through the TCR. Since T cell differentiation and thymic selection appear to require TCR-mediated signaling, the emergence of $CD4^+$ T cells with TCRs that cannot signal requires explanation. Here, we show that thymocytes from a ZAP-70–deficient patient can indeed signal through the TCR. This is in marked contrast to the patient's peripheral T cells, which fail to respond to TCR stimuli. Using a human T lymphotropic virus (HTLV-1)–transformed thymocyte line from this patient, we provide evidence that Syk may compensate for the loss of ZAP-70 in the thymus. These results emphasize the critical but perhaps redundant role for this family of PTKs in T cell differentiation and selection.

Materials and Methods

Cells and Antibodies. PBMCs were isolated by density gradient centrifugation from the patient and normal volunteers. A thymic biopsy was obtained from the patient after informed consent from a parent. A portion of normal thymus was obtained from a patient undergoing cardiac surgery. Single cell suspensions from the thymus were obtained by density gradient centrifugation. HTLV-1 transformation of thymocytes from the patient and from an immunologically normal individual undergoing cardiac surgery was performed as described (32). The HTLV-1 lines were maintained in RPMI 1649 medium supplemented with antibiotics, glutamine, FBS (10%), and IL-2 (20 U/ml). The phenotype of the lines was similar; they each expressed the TCR-α/β (no TCR-δ was detected), CD4, and CD25, but they were CD8 deficient.

The following mAbs were used in these studies: anti-Leu4 (anti-CD3), anti-α/β (WT31), and biotin derivatives (Becton Dickinson & Co., Mountain View, CA); anti–Leu3-biotin (anti-CD4; Becton Dickinson); 4G10 (antiphosphotyrosine) and anti–phospholipase-γ1 (anti–PLC-γ1) (Upstate Biologicals, Inc., Lake Placid, NY); and anti–TCR-δ (T-Cell Diagnostics, Woburn, MA); anti-Lck (a kind gift from Dr. J. Bolen); anti-CD3 ϵ (DAKO, Santa Barbara, CA); a mix of mAbs G3/B2, anti–TCR-ζ (33); and 2F3, anti–ZAP-70 (23). Rabbit heterosera used included: anti–ZAP-70 (26); anti-Fyn (a kind gift from Dr. J. Bolen, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ); anti-Syk (a kind gift from Dr. A. DeFranco, University of California, San Francisco, CA); anti–TCR-ζ (a kind gift from Dr. A. Weissman, National Institutes of Health, Bethesda, MD); and anti-FcRγ (a kind gift from Dr. J. P. Kinet, National Institutes of Health, Bethesda, MD).

Flow Cytometric Analysis of Immunofluorescent Staining of PBMC and Thymocytes. Standard direct immunofluorescent methods were used to double stain for CD4 and CD8 expression on PBMC and thymocytes using antibodies from Coulter Corp. (Hialeah, FL). The cells were analyzed on an Epics Profile I flow cytometer (Coulter).

Changes in $[Ca2^+]_i$. After the loading of PBMCs or thymocytes with indo-1 (Molecular Probes, Eugene, OR) (1 μg/ml), changes in $[Ca^{2+}]_i$ in response to Leu 4 mAb (anti-CD3) were monitored in a spectrofluorimeter (F4010; Hitachi, Tokyo, Japan) and calculated as previously described (34). Changes in $[Ca^{2+}]_i$ in indo-1–loaded, HTLV-1–transformed thymocyte lines were monitored in a SPEX spectrofluorimeter at 37°C according to previously published methods (9). Cells were stimulated with biotinylated anti-Leu4 mAb (2 μg/ml) followed by the addition of streptavidin (10 μg/ml; Calbiochem, La Jolla, CA).

Immunoprecipitation and Western Blot Analyses. Methods are identical with those previously described in detail (18, 22, 23), with the exception that in some experiments enzyme-linked chemoluminescence (ECL) was used to detect proteins by Western blotting. Cells were stimulated in PBS at 37°C at a concentration of 10^8 cells per ml with biotinylated anti-Leu4 mAb and streptavidin. In some experiments, biotinylated anti-Leu3 mAb was also included. Cells were lysed in a buffer containing 1% NP-40. Whole cell lysates from $1–2 \times 10^6$ unstimulated or stimulated cells were diluted in SDS before analysis by Western blotting. For immunoprecipitations, lysates derived from $1–5 \times 10^7$ cells were used.

Northern Blot Analysis. Whole cell RNA was obtained using RNAsol (Teltest, Inc., Friendswood, TX). 30 μg of RNA was electrophoresed in a 1% agarose gel and transferred to nylon membranes. Duplicate filters were hybridized with either a full-length ZAP-70 cDNA probe or a β-actin probe that had been radiolabeled with ^{32}P (26). Washed blots were assessed by autoradiography.

Results

Phenotype of Peripheral Blood T Lymphocytes and Thymocytes. The patient was a 4 1/2-mo-old white male with a 2-mo history of multiple infections and chronic diarrhea. A first child, he was the product of a consanguineous relationship. Initial evaluation revealed panhypogammaglobulinemia and normal numbers of circulating lymphocytes.

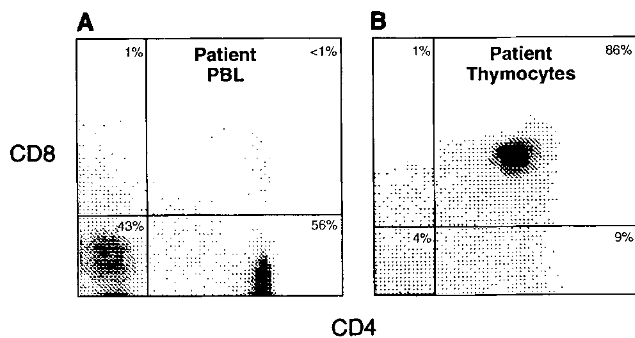

Figure 1. Deficiency of $CD8^+$ T cells in the peripheral blood and thymus of a patient with SCID. PBMC (*A*) and thymocytes (*B*) were obtained and stained with anti-CD4 (phycoerythrin) and anti-CD8 (FITC) mAb, and they were analyzed by flow cytometry.

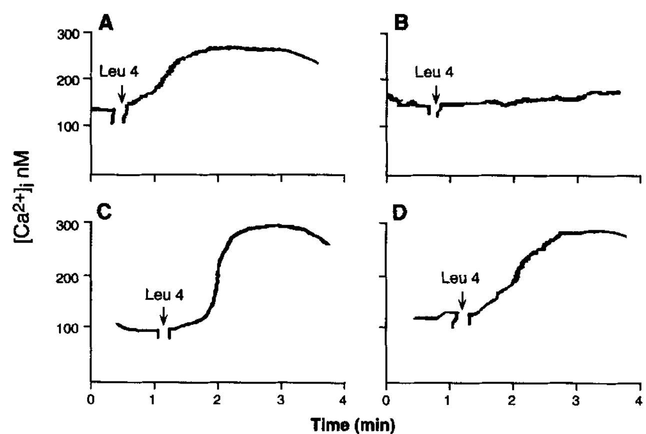

Figure 2. The TCR on the thymocytes but not on the peripheral blood T cells can induce increases in $[Ca^{2+}]_i$. Indo-1–loaded PBMCs from a normal individual (*A*), PBMCs from the patient (*B*), thymocytes from a normal individual (*C*), or thymocytes from the patient (*D*) were stimulated with anti-CD3 mAb as indicated, and changes in $[Ca^{2+}]_i$ were monitored by fluorimetry over time.

Examination of peripheral blood T cells revealed adequate numbers of $CD4^+$ T cells but a marked paucity of $CD8^+$ T cells (Fig. 1 *A*); very few $CD3^+$ $CD8^+$ cells could be detected (<1%). Approximately 40% of peripheral blood MNC were $CD19^+$ B cells. T cell proliferative responses and IL-2 production to a variety of stimuli (phytohemagglutinin, anti-CD3, anti-CD2, anti-TCR) were virtually absent. In contrast, the combination of phorbol ester/ionomycin triggered a normal, IL-2–dependent proliferative response (Mazer, B.M., A. Hayward, and E. W. Gelfand, manuscript in preparation).

At ~6 mo of age, a thymus biopsy was performed, revealing essentially normal architecture and the presence of Hassall's corpuscles. Single-cell preparations were analyzed (Fig. 1 *B*). The majority of thymic cells were doubly stained for CD4 and CD8, ~9% were single $CD4^+$, and few if any $CD8^+$ single positive were detected.

Signaling through the TCR Mediates Changes in $[Ca2^+]_i$ in Thymocytes, but not in Peripheral T Cells in this Patient. The immunologic studies suggested that this patient's $CD4^+$ peripheral blood T cells might have impaired signal transduction function through the TCR. Changes in $[Ca^{2+}]_i$ after TCR stimulation with an anti-CD3 mAb were therefore examined by fluorimetry. After stimulation with anti-CD3 mAb, a rapid and sustained increase in $[Ca^{2+}]_i$ was observed in T cells from a normal individual's PBMCs (Fig. 2 *A*). In contrast, no changes in $[Ca^{2+}]_i$ were observed in the patient's PBMCs (Fig. 2 *B*). Similarly, unlike the responses observed in cells from normal individuals, stimulation of this patient's PBMCs with anti-CD2 mAbs or phytohemagglutinin also failed to induce increases in $[Ca^{2+}]_i$ (data not shown). However, addition of aluminum fluoride to the patient's cells, which mobilizes $[Ca^{2+}]_i$ through mechanisms involving guanine nucleotide–binding proteins, induced a rapid and sustained increase in $[Ca^{2+}]_i$ (data not shown). This latter result suggests that the phosphatidylinositol signal transduction pathway, which regulates increases in $[Ca^{2+}]_i$ induced by the TCR, albeit through a distinct PLC, is intact in the patient's cells. Collectively, these observations demonstrate that the TCRs expressed by the $CD4^+$ T cells in this patient were unable to couple to TCR-regulated mechanisms responsible for increases in $[Ca^{2+}]_i$.

In view of the paradoxical presence of CD4 cells with impaired TCR signal transduction function in the peripheral blood of this patient, the signal transduction function of TCRs on the thymocytes from this patient was of great interest. In contrast to the PBMCs from this patient, addition of anti-CD3 antibody to thymocytes isolated from the biopsy specimen from this patient resulted in a rapid and sustained increase in $[Ca^{2+}]_i$ (Fig. 2 *D*). This increase in $[Ca^{2+}]_i$ was similar in magnitude to the changes observed in thymocytes isolated from a normal individual (Fig. 2 *C*). Indeed, in additional experiments using flow cytometry to monitor calcium changes in individual cells, >80% of the patient's thymocytes responded to the anti-CD3 mAb. Since the patient's thymocyte population was predominantly composed of $CD4^+/CD8^+$ (double-positive) immature thymocytes (86%), these studies suggest that TCRs expressed on these immature double positive thymocytes were able to mediate early signal transduction events and increases in $[Ca^{2+}]_i$.

ZAP-70 is Deficient in the Patient's PBMCs and in a Thymic Line Derived from the Patient. To define the molecular basis for the TCR signal transduction defect resulting in differences in the signaling capacity of this patient's PBMCs and thymocytes, we analyzed several PTKs implicated in TCR signaling. As shown in Fig. 3 *A*, normal levels of Lck were detected in whole-cell lysates of PBMCs from this patient, but no ZAP-70 protein was detected by Western blotting using an mAb directed against the NH_2-terminal region encompassing the SH2 domains of ZAP-70.

To define the molecular basis for the failure of this patient's peripheral blood $CD4^+$ cells to express ZAP-70 protein, and to understand the differential signaling capacity of TCRs in the thymus and PBMCs, an HTLV-1–trans-

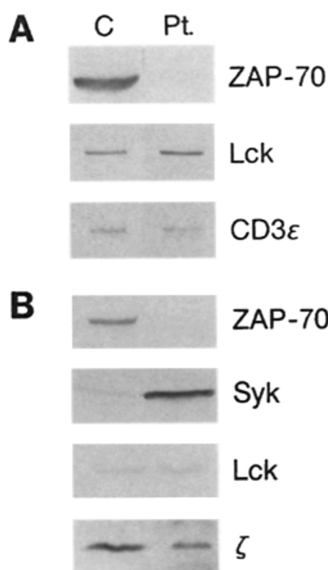

Figure 3. Absence of detectable ZAP-70 protein in the patient's peripheral blood T cells and in an HTLV-1–transformed thymocyte derived from the patient. Whole-cell lysates from PBMCs (*A*) or from HTLV-1–transformed lines derived from thymocytes (*B*) isolated from the patient (*Pt*) or from immunologically normal individuals (*C*) were probed by Western blot analysis for the indicated proteins. Equivalent numbers of cells were used in each of these experiments.

formed polyclonal cell line was derived from the thymocytes of this patient as well as from the thymocytes of an immunologically normal individual undergoing cardiac surgery. Like the patient's peripheral blood $CD4^+$ T cells, the patient's $CD4^+$ thymocyte line also failed to express ZAP-70 protein as assessed by Western blot analysis (Fig. 3 *B*). This is in marked contrast to the ZAP-70 protein easily detected in lysates from the control thymocyte line. The patient's HTLV-1 thymocyte line expressed Lck and TCR-ζ (Fig. 3 *B*), as well as CD4, Fyn, PLC-γ1, and CD3ε (data not shown). Despite the somewhat reduced levels of TCR-ζ, the TCR level on the plasma membrane of the patient's line was comparable to that of the control line. Interestingly, the patient's HTLV-1 line expressed higher levels of Syk when compared to the control line. This was verified in separate experiments using independently derived anti-Syk rabbit heterosera. We were unable to analyze levels of Syk protein in the peripheral blood T cells from the patient since limited quantities of peripheral blood were available and Syk is expressed at high levels in monocytes and B cells, but only at very low levels in normal T cells.

The failure of this patient's PBMCs to mediate increases in $[Ca^{2+}]_i$, together with the deficiency in ZAP-70 protein, is consistent with recent reports of patients with a similar peripheral blood T cell phenotype and signal transduction defect (7, 9). Previous studies (7–9) demonstrated mutations in the coding sequence of the ZAP-70 kinase domain in several independent families. In an effort to further define the molecular basis for the failure of the patient's T cells to express ZAP-70 protein, Northern blots were prepared with whole-cell RNA isolated from the patient and control thymocyte lines. In contrast to previous reports of ZAP-70–deficient patients, no ZAP-70 RNA could be detected in the thymocyte line derived from the patient (Fig. 4). The consanguinity present in this family is likely to have contributed to this defect resulting from the homozygous inheritance of two alleles of ZAP-70 that are transcriptionally silent. Further molecular analysis of this patient's ZAP-

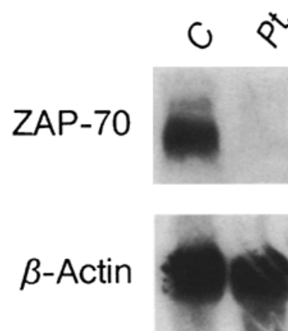

Figure 4. The patient's thymocyte line fails to express ZAP-70 transcripts. Total cellular RNAs derived from the patient's (*Pt*) or the control's (*C*) HTLV-1–transformed thymocyte line were assessed by Northern blot analysis for ZAP-70 or β-actin transcripts.

70 gene is in progress but preliminary Southern blot analysis has failed to reveal gross deletions in the ZAP-70 gene (data not shown). Collectively, these studies suggest that the TCR on $CD4^+$ peripheral T cells from this patient fail to signal because of the absence of ZAP-70 transcripts and protein.

The TCR on the Patient's Thymocyte Line Is Competent to Mediate Signal Transduction Events. In an effort to further understand the molecular basis for the differential signal transduction capability of the TCR on the patient's thymocytes and peripheral T cells, the signal transduction function of the TCR on the HTLV-1–transformed thymocyte line was analyzed by examining changes in $[Ca^{2+}]_i$. The control and patient lines were rested for 16 h in the absence of exogenous IL-2 before stimulation of the TCR with anti-CD3 mAb. Similar to the response of the TCRs on thymocytes from the patient's biopsy (Fig. 2 *D*), the TCR on the patient's HTLV-1–transformed thymocyte line responded to anti-CD3 mAbs, with increases in $[Ca^{2+}]_i$ (Fig. 5 *B*), although somewhat delayed when compared to those of the control thymocyte (Fig. 5 *A*). The patient's thymocyte line thus appears to express a TCR which, like the freshly isolated thymocytes from this patient, is able to induce a signal transduction event characteristic of the TCR on normal T cells and thymocytes.

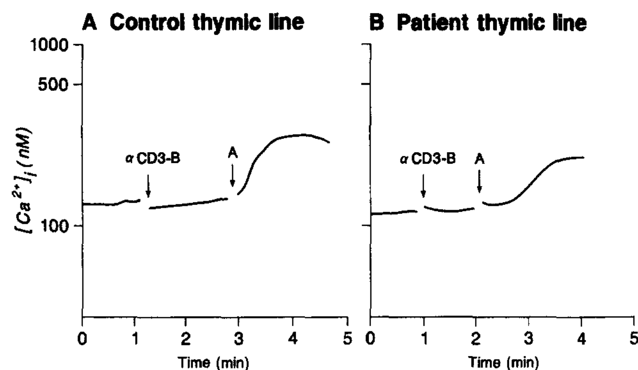

Figure 5. The TCR on the patient's HTLV-1–transformed thymocyte line can induce increases in $[Ca^{2+}]_i$. Indo-1–loaded control (*A*) or patient (*B*) HTLV-1–transformed thymocyte lines were stimulated with biotinylated anti-CD3 mAb (Leu4-B) followed by streptavidin (*A*) as indicated. Changes in $[Ca^{2+}]_i$ were monitored by fluorimetry.

Induction of Tyrosine Phosphoproteins after Stimulation of the TCR on the Thymic Line. Because of the limited patient material available, signal transduction function of the TCR was studied in more detail with the available HTLV-1–transformed thymocyte line. Since previous reports of ZAP-70–deficient peripheral T cells demonstrated a failure of these cells to mediate an increase in the tyrosine phosphorylation of several cellular substrates, including PLCγ1 (7, 9), we examined whole-cell lysates from control and patient HTLV-1–transformed thymocyte lines after stimulation with anti-CD3 mAb. Similar to the rapid induction of tyrosine phosphoproteins observed in the control thymocyte line, increases in tyrosine phosphoproteins could be observed after stimulation of the TCR on the patient thymocyte line (Fig. 6 *A*). Virtually identical results were obtained regardless of whether the TCR was cross-linked with anti-CD3 mAb alone or after co–cross-linking with a combination of anti-CD4 and anti-CD3 mAbs (data not shown). Interestingly, the pattern of tyrosine phosphoproteins in the patient's thymocyte line differed from the control thymocyte line. Notably, prominent 70- and 72-kD tyrosine phosphoproteins were observed in the control line, while only a 72-kD tyrosine phosphoprotein was abundant in the patient line. In addition, a protein migrating at ~24 kD was observed in the control line but not in the patient line. This phosphoprotein may represent a TCR-ζ chain and/or CD3 components (see below). These results suggest that the TCR in the patient thymocyte line is able to couple to cytoplasmic PTKs.

Induction of tyrosine phosphoproteins after TCR stimulation of the patient's thymocyte line was somewhat surprising since previous studies of the TCR in peripheral blood T cell from patients with ZAP-70 deficiency had shown a relative failure to induce tyrosine phosphoproteins after receptor stimulation (7–9). The ability of the patient's thymocytes and the HTLV-1–transformed thymic line to signal in the absence of ZAP-70, was further assessed by immunoprecipitation of Syk, ZAP-70, and the ζ chain from the control and patient thymic lines before and after TCR stimulation. Stimulation of the TCR in the control line resulted in the induction of prominent tyrosine phosphorylation of ZAP-70, as well as the ζ chain (Fig. 6, lanes *10* and *14*), but relatively minimal induction of Syk tyrosine phosphorylation (Fig. 6, lane *6*). ZAP-70 immunoprecipitates from the control line contained only low levels of tyrosine-phosphorylated ζ chain in the basal state (Fig. 6, lane *9*) but an increased level from stimulated cells (Fig. 6, lane *10*). In marked contrast, no tyrosine phosphorylation of the ζ chain was observed in Syk immunoprecipitates, despite the modest induction of tyrosine phosphorylation of Syk in the control line. Prolonged exposures of this blot failed to demonstrate an association between Syk and the ζ chain or CD3 chains in the control line. Thus, in the control line, there was a marked increase in the tyrosine phosphorylation of ζ and ZAP-70 but only a modest increase in Syk phosphorylation. No association between Syk and ζ was observed after TCR stimulation.

In contrast to the response in the control line, Syk was more highly phosphorylated after TCR stimulation of the patient thymocyte line (Fig. 6, lane *8*). These findings suggest that Syk undergoes very prominent phosphorylation in the absence of ZAP-70. In part, this could reflect the higher levels of Syk expression in the patient line (Fig. 3 *B*). Despite this marked degree of tyrosine phosphorylation of Syk, no associated ζ chain was coimmunoprecipitated with Syk, nor was the ζ chain detectably tyrosine phosphorylated in the patient line (Fig. 6, lanes *8* and *16*). Thus, Syk does not appear to form a stable complex with ζ, nor could we detect the induced tyrosine phosphorylation of ζ. In other experiments, we were also unable to detect tyrosine phosphorylation of the CD3 chains (including CD3ε) in the patient line after TCR stimulation (data not shown). Moreover, we were unable to detect the FcRγ chain in lysates of the thymic cell line by immunoblotting (in contrast to a rat basophilic leukemia cell line as a control, data not shown), eliminating the possibility of Syk association with the TCR via phosphorylated FcRγ. These findings suggest that Syk can couple to the TCR signaling pathway in the absence of ZAP-70, though its interaction with the TCR may differ qualitatively or quantitatively.

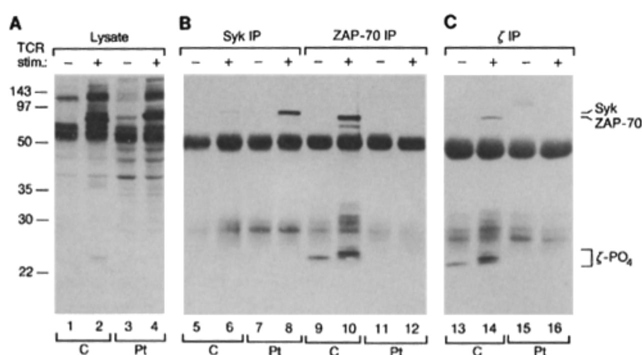

Figure 6. Stimulation of the TCR on the patient's thymocyte line induces increases in cellular tyrosine phosphoproteins including Syk. (*A*) Whole-cell lysates from the control thymocyte line (*C*) or from the patient's thymocyte line (*Pt*) were prepared before (−) or after stimulation with biotinylated anti-CD3 mAb and streptavidin (+) and blotted with an antiphosphotyrosine mAb. (*B*) Syk (lanes *5–8*) or ZAP-70 (lanes *9–12*) immunoprecipitates were isolated using rabbit heterosera from the lines before or after stimulation and blotted with an antiphosphotyrosine mAb. (*C*) TCR-ζ immunoprecipitates (lanes *13–16*), isolated from control and patient lines before and after stimulation using an anti–TCR-ζ heteroserum, were probed with an antiphosphotyrosine mAb. Note that the band running below ZAP-70 in lane *10* likely represents Lck, since Lck and ZAP have been shown to coprecipitate with the ζ chain.

Discussion

At least four cytoplasmic PTKs have been shown to interact with the TCR and to contribute to its signal transduction function: Fyn, Lck, ZAP-70, and Syk. The consequences of mutations in three of these PTKs have resulted in rather distinct phenotypes in mice whose genes have been purposefully disrupted, or in humans whose disease results from inherited mutations. The resultant phenotypic

abnormalities provide unique insights into the role of TCR signal transduction and these PTKs in TCR function and in T cell development.

In mice lacking *fyn* as a result of gene disruption, general features of T cell subset maturation are not altered and the numbers of T cells in the thymus, and peripheral lymphoid organs are preserved (16, 17). The loss of Fyn has the greatest impact in TCR signal transduction function within the most mature single positive ($CD4^+$ or $CD8^+$) subsets of thymocytes, whereas the signal transduction function of TCRs on peripheral T cells appears to be restored to a large extent (16, 17). There is an impairment in signal transduction through the GPI-linked molecule Thy-1 (17). These observations suggest that the function of Fyn in TCR signaling may be most important in a developmentally restricted compartment or in signal transduction via receptors other than the TCR.

In contrast to *fyn*, disruption of the *lck* gene results in a severe arrest of thymocyte development at a very immature stage, the transition from double negative ($CD4^-/CD8^-$) to double positive ($CD4^+/CD8^+$) thymocytes (20). A small number of T cells are found in the peripheral lymphoid tissues. Whether this occurs via compensatory upregulation of other PTKs was not examined. The few peripheral T cells that do develop and populate peripheral lymphoid organs also exhibit diminished responses to typical TCR stimuli, suggesting an impairment in TCR signal transduction function. This is consistent with the loss of TCR signal transduction function in a Jurkat-derived T cell mutant line and in a cytolytic T cell clone that were found to be deficient in Lck kinase expression (18, 19). Lck also appears to play a role in allelic exclusion of the TCR-β chain (35). These observations suggest that Lck plays a prominent role in TCR signal transduction and contributes to developmental decisions early during thymic ontogeny.

In humans, mutations in the ZAP-70 gene that impair expression or function give rise to a phenotype that is clearly distinct from the deletions of *fyn* or *lck*. The peripheral blood and thymus are deficient in $CD8^+$ T cells, and circulating $CD4^+$ T cells fail to signal appropriately through the TCR. There is a failure of protein tyrosine phosphorylation and the mobilization of $[Ca^{2+}]_i$ after TCR stimulation, suggesting that ZAP-70 is essential for TCR-mediated signaling in $CD4^+$ cells. Moreover, it would appear that redundant pathways or compensatory changes in the functions of other PTKs are not present or certainly not detectable in the ZAP-70–deficient peripheral $CD4^+$ T cells. Since there are abundant numbers of double positive (cortical) and $CD4^+$ (medullary) thymocytes with a paucity of $CD8^+$ (medullary) thymocytes, this also implies that ZAP-70 may not be essential for the transition of double-positive $CD4^+/CD8^+$ thymocytes to single positive $CD4^+$

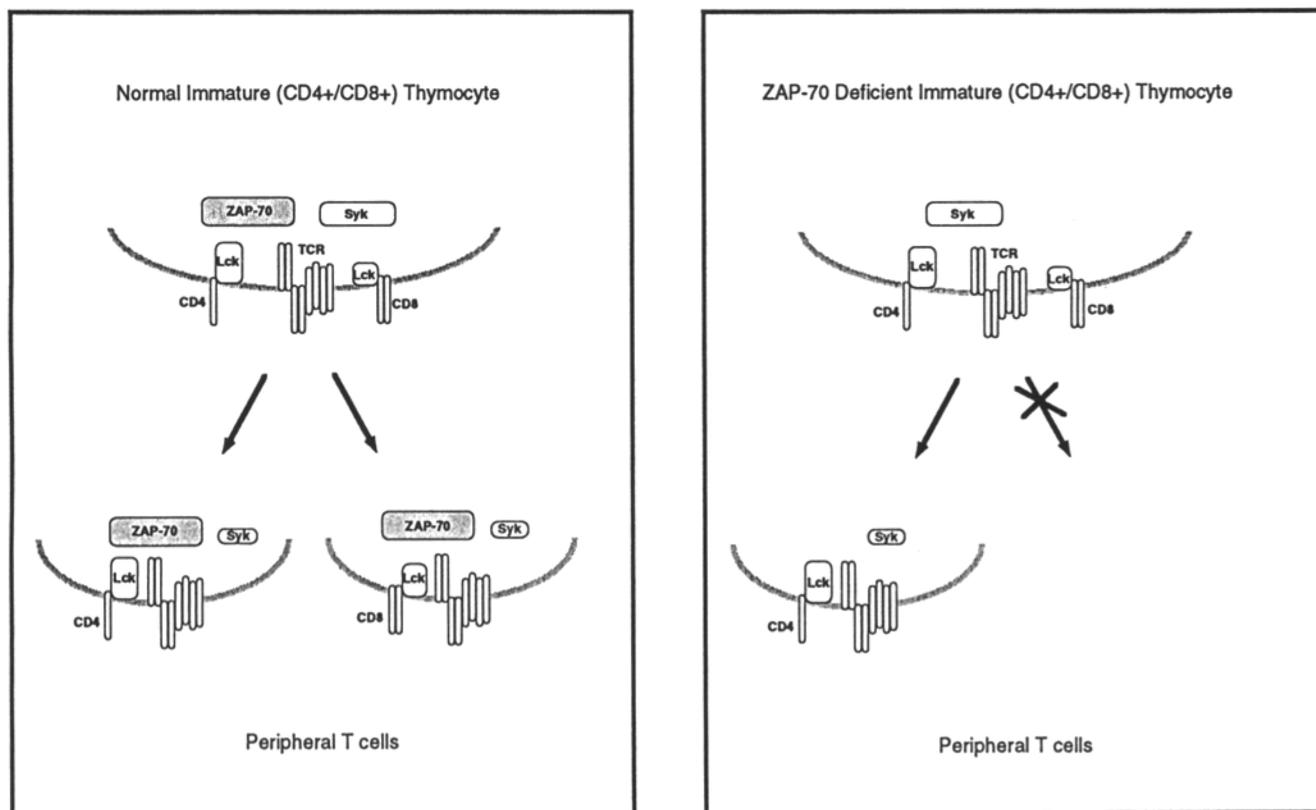

Figure 7. Scheme for T cell selection in the absence of ZAP-70. ZAP-70 may be indispensable for maturation into $CD8^+$ T cells but not $CD4^+$ T cells. For $CD4^+$ T cell selection in the thymus, Syk may be capable of compensating for the loss of ZAP-70. In addition, the differential level of association of Lck with CD4 and CD8 is shown.

T cells. Based on the phenotype of the ZAP-70–deficient patients, it appears that ZAP-70 may be indispensable for maturation into $CD8^+$ T cells, but not $CD4^+$ T cells (see model in Fig. 7).

The homologous 72-kD PTK Syk may also play a role in TCR signal transduction. As observed for the stimulation of ZAP-70, TCR stimulation results in the tyrosine phosphorylation of Syk and the association of phosphorylated ζ with Syk in the Jurkat T lymphoblastoid cell line and in a heterologous Cos cell expression system (22). Moreover, chimeras in which Syk has been fused to another transmembrane receptor can initiate signal transduction events similar to those induced by the stimulated TCR (36). Thus, Syk may play a role in TCR signal transduction that is similar, though perhaps not identical to that of ZAP-70. In the thymus, where expression of Syk is highest in the T cell lineage (22), Syk may be capable of compensating for the loss of ZAP-70. This may account for the ability of the TCR on thymocytes in ZAP-70–deficient individuals to mediate signal transduction events. This could, in turn, permit the $CD4^+$ lineage cells to be positively selected and circulate in the peripheral blood (Fig. 7).

The evidence that Syk is coupled to the TCR and can compensate for the loss of ZAP-70 in the thymus of these patients is supported by our observations of the patient's HTLV-1–transformed thymocyte line which, like the fresh thymocytes from this patient, signaled through the TCR. This suggests that the TCR in this line is coupled to cytoplasmic PTKs. After TCR stimulation, Syk was rapidly tyrosine phosphorylated in this ZAP-70–deficient cell. This also occurred in the control thymocyte cell line, but not to the same extent. The smaller degree of tyrosine phosphorylation of Syk in the control line could reflect its competition with ZAP-70 for interaction with the TCR. Alternatively, it could reflect the lower levels of Syk expression in the ZAP-70–positive control line. The higher level of expression of Syk in the ZAP-70–deficient cells might represent a compensatory mechanism in response to ZAP-70 loss. Unfortunately, this could not be confirmed with fresh ZAP-70–deficient thymocytes from this patient since they were not available for analysis. The HTLV-1–transformed thymocyte cell line from the patient resembled his thymocytes in that the levels of Syk were higher than in peripheral T cells. The downregulation of Syk expression that occurs in peripheral T cells, as has been described in normal humans and mice, could account for the loss of TCR signal transduction function in the peripheral blood $CD4^+$ T cells in ZAP-70–deficient patients.

If Syk compensates for the loss of ZAP-70 in the thymus, the failure of Syk to participate in the positive selection of $CD8^+$ cells must be explained. One possibility is that differentiation towards the CD4 rather than the CD8 lineage reflects the involvement of a more efficient signal transduction mechanism. If Syk is unable to fully replace the function of ZAP-70, signal transduction by the TCR in the thymus might be partially compromised. Since Lck has a greater association with CD4 than CD8 in double-positive thymocytes, this may contribute to a preferential selection of the CD4 lineage (37). Thus, signal transduction by the TCR in ZAP-70–deficient thymocytes may only reach an appropriate threshold in the events leading to CD4 but not CD8 lineage selection.

An unexpected finding in these studies was the failure to detect tyrosine-phosphorylated Syk associated with ζ or CD3 after TCR stimulation in either of the HTLV-1 thymocyte lines. We also failed to detect any phosphorylated ζ or CD3 chains in the ZAP-70–deficient line after TCR stimulation despite the inducible tyrosine phosphorylation of Syk and other proteins. It is possible that the interaction of Syk with the TCR is a transient event in these HTLV-1 lines. Alternatively, these lines might express regulatory proteins that cause a rapid dissociation of the SH2 domains of Syk from the phosphorylated tyrosine residues contained in the activation motifs present in the ζ and CD3 chains. Another possibility is raised by the studies of Couture et al. (31), who suggested that Syk might function upstream of Lck and ZAP-70. Syk levels, however, are very low in peripheral T cells where signaling does not appear to occur in the absence of ZAP-70, and we have not been able to reproduce their findings (data not shown).

Our studies provide insights into mechanisms that may explain the paradoxical phenotype of peripheral T cells in ZAP-70–deficient patients. The ability of the TCR to differentially signal in the thymus but not in peripheral $CD4^+$ cells offers an explanation for how $CD4^+$ cells are selected. Our results suggest that in the absence of ZAP-70, Syk may play a compensatory role in regulating this positive selection (Fig. 7). The preferential selection of $CD4^+$ cells may reflect an increased association of Lck with CD4 rather than CD8. Thus, in a cell with a compromised TCR signaling pathway because of ZAP-70 deficiency, only the selection events that involve CD4 coreceptors may yield signals of sufficient magnitude for further differentiation into more mature cells. The absence of single positive CD8 cells in the thymus of these patients supports the notion that positive selection occurs at the transition of double-positive to single-positive cells. Downregulation of Syk during development and/or the absolute requirement for ZAP-70 may explain why peripheral $CD4^+$ T cells lacking ZAP-70 do not have signal competent TCRs.

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Address correspondence to Erwin Gelfand, M.D., Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Diseases, 1400 Jackson Street, Denver, CO 80206.

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