



HAL
open science

Alternative antigen receptor (TCR) signaling in T cells derived from ZAP-70-deficient patients expressing high levels of Syk

N. Noraz, K. Schwarz, M. Steinberg, V. Dardalhon, C. Rebouissou, R. Hipskind, W. Friedrich, H. Yssel, K. Bacon, N. Taylor

► To cite this version:

N. Noraz, K. Schwarz, M. Steinberg, V. Dardalhon, C. Rebouissou, et al.. Alternative antigen receptor (TCR) signaling in T cells derived from ZAP-70-deficient patients expressing high levels of Syk. *Journal of Biological Chemistry*, 2000, 275 (21), pp.15832–8. 10.1074/jbc.M908568199 . hal-02196824

HAL Id: hal-02196824

<https://hal.science/hal-02196824>

Submitted on 27 May 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

Alternative Antigen Receptor (TCR) Signaling in T Cells Derived from ZAP-70-deficient Patients Expressing High Levels of Syk*

Received for publication, October 20, 1999, and in revised form, March 13, 2000
Published, JBC Papers in Press, March 14, 2000, DOI 10.1074/jbc.M908568199

Nelly Noraz^{‡§}, Klaus Schwarz[¶], Marcos Steinberg^{‡||}, Valérie Dardalhon[‡], Cosette Rebouissou[‡], Robert Hipskind[‡], Wilhelm Friedrich^{**}, Hans Yssel^{‡‡}, Kevin Bacon^{§§¶¶}, and Naomi Taylor[‡]

From the [‡]Institut de Génétique Moléculaire de Montpellier, CNRS UMR 5535, 1919 Route de Mende, 34293 Montpellier, Cedex 5, France, the Departments of [¶]Transfusion Medicine and ^{**}Pediatrics, University of Ulm, D89081 Ulm, Germany, the ^{‡‡}INSERM U454, 34295 Montpellier, France, and the ^{§§}Department of Immunology, Neurocrine Biosciences Inc., San Diego, California 92121

ZAP-70-deficient patients present with nonfunctional CD4+ T cells in the periphery. We find that a subset of primary ZAP-70-deficient T cells, expressing high levels of the related protein-tyrosine kinase Syk, can proliferate *in vitro*. These cells (denoted herein as Syk^{hi}/ZAP-70⁻ T cells) provide a unique model in which the contribution of Syk to TCR-mediated responses can be explored in a nontransformed background. Importantly, CD3-induced responses, such as tyrosine phosphorylation of cellular substrates (LAT, SLP76, and PLC- γ 1), as well as calcium mobilization, which are defective in T cells expressing neither ZAP-70 nor Syk, are observed in Syk^{hi}/ZAP-70⁻ T cells. However, Syk^{hi}/ZAP-70⁻ T cells differ from control T cells with respect to the T cell antigen receptor (TCR)-mediated activation of the MAPK cascades: extracellular signal-regulated kinase activity and recruitment of the JNK and p38 stress-related MAPK pathways are diminished. This distinct phenotype of Syk^{hi}/ZAP-70⁻ T cells is associated with a profound decrease in CD3-mediated interleukin 2 secretion and proliferation relative to control T cells. Thus, ZAP-70 and Syk appear to play distinct roles in transducing a TCR-mediated signal.

Severe combined immunodeficiency syndrome comprises a group of genetic disorders affecting T and B lymphocyte function. One variant of severe combined immunodeficiency syndrome is caused by an absence of ZAP-70, a protein-tyrosine kinase that is recruited to the phosphorylated ζ chain of the TCR following its stimulation (1). ZAP-70-deficient patients are characterized by a selective inability to produce CD8+ T cells and an inability of mature CD4+ T cells to respond to TCR stimulation (2–4). Thus, ZAP-70 appears to play a critical role in T cell ontogeny as well as T cell activation. It is interesting to note that the phenotype of ZAP-70-deficient mice and humans are distinct, with ZAP-70-mutant mice exhibiting an earlier block in T cell development, at the CD4+CD8+ thymo-

cyte stage (5, 6). Although the bases for the differential role of ZAP-70 in human and murine T cell development remain unclear, it is likely that compensatory mechanisms exist in ZAP-70^{-/-} patients that allow CD4+ T cells to mature and emigrate to the periphery. One potential substitute for ZAP-70 is the structurally homologous Syk protein-tyrosine kinase. The compensatory role of Syk is likely more pronounced in the thymus than in peripheral T cells because it is down-regulated during T cell development (7). Indeed, a TCR-induced response can be elicited in Syk-expressing thymocytes derived from a ZAP-70-deficient patient but not in peripheral CD4+ T cells from the same individual (8).

It remains important to test whether the differential activation of ZAP-70 and Syk participates in the modulation of a T cell response. In favor of distinct roles for ZAP-70 and Syk in T cell activation are the observations that Syk kinase activity is 100-fold higher than that of ZAP-70 (9) and that the ability of Syk to be activated in an Lck-independent fashion is not shared by ZAP-70 (10–14). Finally, studies of a recently established ZAP-70⁻/Syk⁻ Jurkat clone (p116) have demonstrated that transient expression of ZAP-70 but not Syk reverses the defect in ζ chain phosphorylation (15). Nevertheless, it has not been possible to use the above-mentioned clone to assess the biological effects of Syk because its stable introduction has not been tolerated. Indeed, there is presently no ZAP-70-deficient T cell model in which Syk is expressed at high levels, making it difficult to assess the precise role of Syk in modulating the response of a T cell to TCR stimulation.

Here, we demonstrate that proliferation of polyclonal populations of nontransformed CD4+ T cells derived from ZAP-70-deficient patients is consistently associated with significant increases in Syk levels, suggesting that Syk substitutes for ZAP-70 in transducing extracellular signals. Indeed, whereas ZAP-70-deficient T cells expressing only low levels of Syk demonstrated a defective response to TCR stimulation, proliferating ZAP-70^{-/-} cells that expressed high levels of Syk (Syk^{hi}/ZAP-70⁻ T cells) exhibited an elevated calcium flux in response to CD3 engagement. However, the ensemble of downstream molecules activated in Syk^{hi}/ZAP-70⁻ T *versus* control T cells was distinct with decreased Erk,¹ JNK, and p38 MAPK activities in the former. This phenotype was associated with a profound defect in TCR-induced proliferation and IL-2 secretion. Thus, although Syk can compensate for ZAP-70 in activating several downstream effector molecules, our data demonstrate

* This work was supported by grants from the Association Française contre les Myopathies, Fondation de la Recherche Médicale (FRM), Association pour la Recherche sur le Cancer (ARC), Ligue Nationale contre le Cancer, INSERM, and CNRS (to N. T.), JZKF.Ulm.CO.5 (to K. S.), and the FRM and ARC (to B. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a fellowship from the Association Française contre les Myopathies. To whom correspondence should be addressed. Tel.: 33-4-67-61-36-28; Fax: 33-4-67-04-02-31; E-mail: noraz@igm.cnrs-mop.fr

¶ Supported by a fellowship from the Fundacion YPF.

¶¶ Present address: Bayer Yakuhin, Ltd., Kyoto 619-0216, Japan.

¹ The abbreviations used are: Erk, extracellular signal-regulated kinase; Ab, antibody; mAb, monoclonal Ab; pAb, polyclonal Ab; IL, interleukin; JNK, c-Jun NH₂-terminal kinase; MBP, myelin basic protein; MAPK, mitogen-activated protein kinase.

that Syk and ZAP-70 are differentially coupled to the TCR signaling cascade.

EXPERIMENTAL PROCEDURES

Cells—The Jurkat T cell line E6-1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and the ZAP-70⁺/Syk⁺ Jurkat clone 77-6.8 was generously provided by Dr. K. A. Smith (New York, NY). Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation from two patients, products of a consanguineous relationship, and normal donors following institutional review board approval and informed consent. CD3⁺/CD4⁺ $\alpha\beta$ T cells were purified using α CD4 coated magnetic beads as per the manufacturer's instructions (Dyna, Inc., Great Neck, NY). Cells were cultured in Yssel's medium (16) supplemented with 1% human AB⁺ serum and recombinant human IL-2 at 100 u/ml (Chiron Corp., Emeryville, CA). Cells were stimulated weekly during the first month in culture and every other week thereafter with PHA (0.5 μ g/ml) (Murex, Dartford, United Kingdom) and irradiated feeder cells consisting of peripheral blood mononuclear cells and Epstein-Barr virus-transformed JY cells as described previously (17). In all experiments described here, α CD3 and IL-2 stimulations were performed on cells in "resting phase" that had not been stimulated with irradiated feeder cells for at least 10 days prior to use. Prior to activation, cells were cultured overnight in Yssel's medium without IL-2.

Antibodies and Flow Cytometry Analysis—An Ab recognizing the dually phosphorylated Thr¹⁸³-Tyr¹⁸⁵ form of Erk1/Erk2 (anti-ACTIVE-MAPK) was obtained from Promega (Charbonnière, France). Abs recognizing the phosphorylated and nonphosphorylated forms of p38 and SAPK/JNK were from New England Biolabs (Beverly, MA). The α Erk2 mAb and α PLC- γ 1 pAb were from Transduction Laboratories (Lexington, KY), and the 4G10 α -phosphotyrosine mAb and the α LAT pAb were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Agarose-conjugated polyclonal α Erk1/Erk2 Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). An α ZAP-70 mAb and polyclonal and monoclonal α Syk Abs were the generous gifts of A. Weiss (University of California, San Francisco, CA). The 9.3 α CD28 mAb was generously provided by C. June (National Institutes of Health, Bethesda, MD). The α CD3 OKT3 hybridoma line was purchased from the ATCC, and the α CD3 UCHT1 mAb was from Pharmingen (San Diego, CA). An α -mouse F(ab')₂ fragment was obtained from Immunotech (Marseille, France). TCR Pan $\alpha\beta$, CD3, CD4, CD8, and IgG isotype control Abs used for FACS analyses were purchased from Immunotech. Standard direct immunofluorescent methods were used for single and double staining of cells for these surface markers. Fluorescence was then examined using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Lymphocyte Proliferation and Cytokine Measurements—Cells (1×10^5 in a total volume of 100 μ l) were cultured in triplicate in flat-bottomed 96-well plates in the presence or absence of exogenous IL-2 (100 u/ml) and immobilized OKT3 mAb (1 μ g/ml). After 3 days in culture, cells were [³H]thymidine-labeled (1 μ Ci/well) (CEA, Saclay, France) for 12 h, harvested, and counted in a scintillation counter (Beckman LS 6000SC). The stimulation index for each cell type was determined by the following formula: [³H]thymidine incorporation in mitogen-treated cells/[³H]thymidine incorporation in nonactivated cells. For measurement of secreted IL-2, cells were activated as described above, and cell-free supernatants were collected 24 h following activation. A commercially available enzyme-linked immunosorbent assay kit for IL-2 (Immunotech) was used according to the manufacturer's instructions.

Calcium Mobilization Analysis—For fluorometry experiments, T cells were loaded with 3 μ M (final) Indo-1-AM (Molecular Probes, Eugene, OR) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 20 °C for 45 min. Cells were then washed and resuspended in *N*- α -Hanks' buffered salt solution (2 nM CaCl₂, 145 nM NaCl, 5 nM KCl, 1 nM MgCl₂, 5 nM D-glucose, 20 nM HEPES, pH 7.3) containing 1% bovine serum albumin and maintained at 20 °C. Cells (5×10^5) were stimulated with the α CD3 UCHT1 mAb (1–5 μ g/ml) at 37 °C in a constantly stirred acrylic cuvette. Fluorescence measurements to determine [Ca²⁺]_i were made using a Photon Technologies fluorometer (PTI, South Brunswick, NJ) with an excitation wavelength of 350 nm (4 nm bandwidth) and dual simultaneous monitoring of emission at 450 and 485 nm (10 nm bandwidth). Results are represented as a ratio of emission at 405/485 nm.

Cell Stimulations and Immunoblots—Cells were washed in PBS, resuspended at 1×10^7 cells/ml, and stimulated with an α CD3 mAb or α CD3/ α CD28 mAbs (10 μ g/ml) followed by cross-linking with an α -mouse F(ab')₂ fragment (40 μ g/ml) at 37 °C for the indicated

time periods.

Cells were lysed in a 1% Nonidet P-40 lysis buffer, and postnuclear supernatants were immunoprecipitated for 1 h at 4 °C with the indicated Ab followed by collection on protein A-Sepharose beads (Amersham Pharmacia Biotech) (18). Immunoprecipitates or whole-cell lysates were boiled, resolved on SDS-polyacrylamide gel electrophoresis gels, and transferred electrophoretically to Hybond-C membranes (Amersham Pharmacia Biotech). Membranes were blocked for 30 min in TBS (150 mM NaCl, 20 mM Tris, pH 7.5) containing 5% bovine serum albumin and 0.1% Tween 20 and incubated with the indicated primary Ab for 1 h at room temperature. Blots were then incubated with horseradish peroxidase-conjugated goat α -rabbit or α -mouse secondary Abs (Amersham Pharmacia Biotech), and immunoreactive proteins were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). For reblotting, filters were stripped as reported (18). In some experiment, bands intensities were quantified using Intelligent Quantifier.

Protein Kinase Assays—An immune complex kinase assay was performed using myelin basic protein (MBP) (Sigma) as a substrate following immunoprecipitation with agarose-conjugated α Erk1 and α Erk2 polyclonal Abs. Immunoprecipitates were washed twice with lysis buffer and once in MAPK wash buffer (25 mM Tris HCl, pH 7.4, 40 mM MgCl₂, 137 mM NaCl, 10% glycerol). Enzyme activity was assessed in a final volume of 20 μ l in a buffer containing 42.5 mM HEPES, 425 mM MgCl₂, 0.21 mM ATP, 50 μ Ci of [γ -³²P]ATP (3000 Ci/mmol) and 50 mM MBP for 30 min at 30 °C. Following separation on an SDS-polyacrylamide gel electrophoresis gel, phosphorylated MBP (molecular mass, 18.4 kDa) was visualized by autoradiography.

RESULTS

Phenotype and Proliferation of Primary ZAP-70-deficient T Cells—T cells were isolated from two siblings who presented with clinical symptoms characteristic of severe combined immunodeficiency. Further analysis revealed a phenotype consistent with a deficiency of the ZAP-70 protein-tyrosine kinase (2–4): normal numbers of CD4⁺ lymphocytes (60%) and a marked defect in CD8⁺ cells (<3%). Similar to observations in other ZAP-70-deficient patients, the patients described here demonstrated a profound defect in T cell proliferation upon TCR and mitogen stimulation (data not shown). We therefore assessed ZAP-70 protein levels and found that it was not expressed in T cells from these two patients (Fig. 1A). DNA sequencing revealed that both patients' ZAP-70 DNA contained a homozygous C to T nucleotide transition at position 1729, resulting in an alanine to valine substitution at amino acid 507 of the kinase domain (Fig. 1B). This newly identified mutation lies within a 13-base pair region that is deleted in another ZAP-70-deficient patient (4). Interestingly, with the exception of a temperature sensitive mutation recently described in the SH2 domain, all other ZAP-70 mutations reported to date are localized in the kinase domain (2–4, 6, 19).

In order to determine whether ZAP-70-deficient T cells could be induced to proliferate, cells from the patients were stimulated under conditions previously described to be optimal for T cell growth (17). Specifically, cells were activated with irradiated allogeneic accessory cells, PHA and exogenous IL-2 in the presence of human serum. Indeed, a subset of ZAP-70⁻/ $\alpha\beta$ CD4⁺ T cells proliferated and the response of these cells to allogeneic restimulation was significantly augmented as compared with fresh T cells isolated from the same patients (not shown). Due to the similarities between Syk and ZAP-70 and the ability of Syk to activate some TCR-induced responses in a transformed Syk⁻/ZAP-70⁻ T cell line (15), we compared Syk levels in fresh and proliferating ZAP-70-deficient and control T cells. The low levels of Syk detected in freshly isolated T cells from these patients, as well as in an unrelated ZAP-70-deficient patient, were associated with a complete block in TCR-induced proliferation (Fig. 2A and data not shown).² Importantly, proliferating ZAP-70-deficient T cells, but not

² C. Hivroz, personal communication.

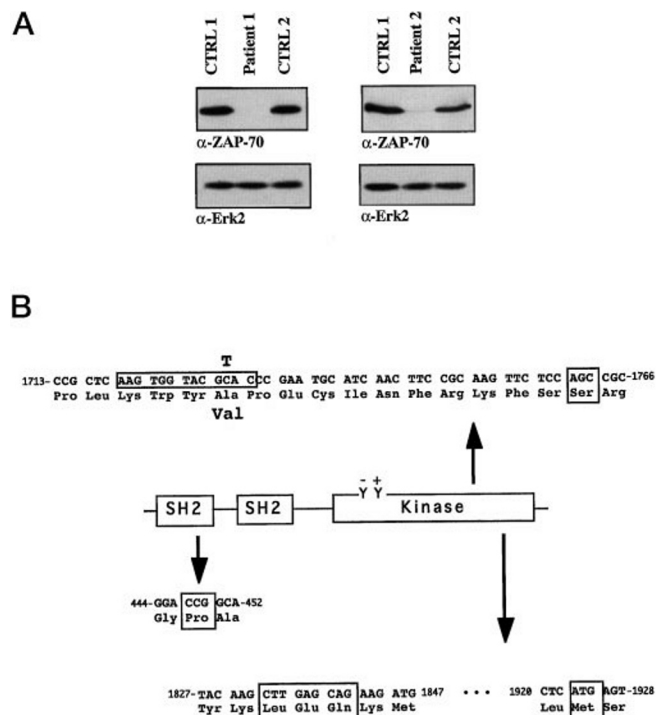


FIG. 1. ZAP-70 deficiency is associated with a mutation at alanine 507 in the kinase domain. *A*, CD4⁺ T cells were isolated from two siblings (patients 1 and 2) and control donors (*CTRL 1* and *CTRL 2*). Total cell lysates (1×10^6 cell equivalents) were fractionated on an SDS gel. Membranes were immunoblotted with an α ZAP-70 mAb and reprobed with an α Erk2 mAb to verify equivalent protein loading. Immunolabeled proteins were visualized by ECL. *B*, the nucleotide sequences corresponding to residues 444–452 (amino acids 79–81), 1713–1766 (amino acids 502–519), 1827–1847 (amino acids 540–546), and 1920–1928 (amino acids 571–573) are shown (1). A homozygous cytidine to thymidine transition at nucleotide position 1729, resulting in the mutation of alanine to a valine at position 507, is indicated. Other mutations located in the kinase domain (*boxed*) include a 13-base pair deletion spanning nucleotides 1719–1731; a point mutation at nucleotide 1763 that changes a serine to an arginine at position 518; a 9-base pair insertion that results in the introduction of a leucine, glutamic acid, and glutamine following amino acid 541; and a point mutation located at base pair 1923 that changes a methionine to a leucine at position 572 (2–4, 19). Additionally, a newly described point mutation in the SH2 domain in which a C to A transition at position 448 results in the substitution of glutamine for proline at residue 80 is indicated (19).

equivalently treated control T cells, expressed significantly higher levels of Syk (Fig. 2*B*). The subsets of proliferating T cells were polyclonal as determined by TCR V β usage (not shown). The augmented Syk expression observed in ZAP-70⁻ T cells was not the result of a selection bias in a single pool of expanded cells, as it was observed in multiple pools from both of the ZAP-70-deficient patients assessed here (Fig. 2*C*) as well as in mature T cells derived from three additional unrelated ZAP-70-deficient patients.^{2,3} It should be noted that the level of Syk observed in T cells obtained from these five ZAP-70-deficient patients was approximately equivalent to that expressed in Syk⁺ Jurkat T cells (Fig. 2 and data not shown). Thus, high Syk expression is a consistent phenotype of proliferating ZAP-70⁻ T cells (designated hereafter as Syk^{hi}/ZAP-70⁻ T cells).

Next, we assessed whether these proliferating Syk^{hi}/ZAP-70⁻ T cells could respond to TCR stimulation alone. Tyrosine phosphorylation of Syk was observed upon CD3 engagement of Syk^{hi}/ZAP-70⁻ T cells but not in equivalently treated control T cells, which expressed Syk at the limits of detection (Fig. 2*C*).

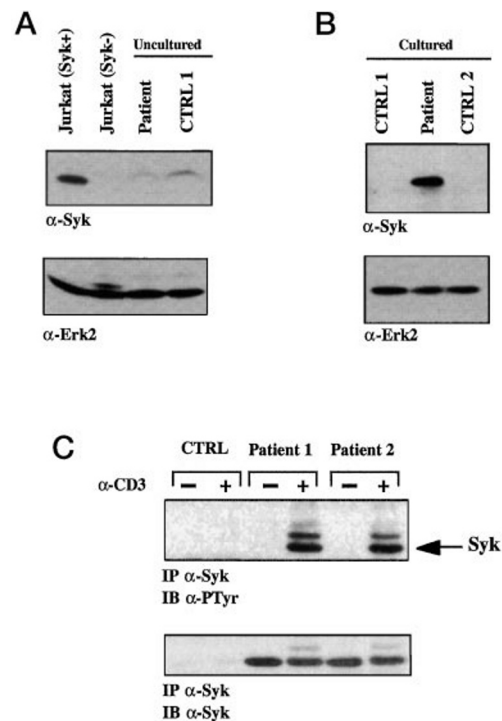


FIG. 2. Syk is highly expressed in proliferating ZAP-70-deficient T cells and is phosphorylated in response to TCR ligation. The level of Syk expression in freshly obtained (*A*) and proliferating (*B*) T cells (1×10^6 cell equivalents) isolated from ZAP-70⁻ patients and control donors was monitored using an α Syk mAb. Immunoblots were then reprobed with an α Erk2 mAb. *C*, Syk was immunoprecipitated from proliferating ZAP-70⁻ T cells from the two patients and identically treated control CD4⁺ T cells (5×10^6 cell equivalents) following no stimulation (-) or stimulation (+) with an α CD3 mAb. Immunoprecipitates (*IP*) were immunoblotted (*IB*) with an α -Tyr(P) (α -PTyr) mAb to assess the phosphorylation status of Syk (*upper panel*). The position of Syk is indicated by an arrow. The blot was then reprobed with an α Syk mAb (*lower panel*).

These results indicate that in Syk^{hi}/ZAP-70⁻ T cells, Syk is likely to be involved in transducing TCR-induced signals from the cell surface.

Tyrosine Phosphorylation of Signaling Intermediates in Syk^{hi}/ZAP-70⁻ T Cells—Significant attention has focused on the mechanisms by which TCR-mediated activation of ZAP-70/Syk kinases are coupled to downstream signaling pathways. LAT (linker for activation of T cells) is a 36/38-kDa membrane-associated adapter protein that has been proposed to link the TCR with some of these downstream events (20). Indeed, LAT associates *in vivo*, either directly or indirectly, with important signaling intermediates, such as PLC- γ 1, Cbl, Vav, SLP76, Grb2, and Grap (20). We therefore assessed the tyrosine phosphorylation of LAT and its association with other signaling molecules in control and Syk^{hi}/ZAP-70⁻ T cells. Cells were stimulated with an α CD3 mAb, and lysates were subjected to an α LAT immunoprecipitation. In Syk^{hi}/ZAP-70⁻ T cells, the level of CD3-induced LAT phosphorylation was equivalent or greater than that observed in control T cells (Fig. 3*A*). In additional experiments, PLC- γ 1 and SLP76 were immunoprecipitated from control and Syk^{hi}/ZAP-70⁻ T cell lysates. Upon CD3 stimulation, PLC- γ 1 and SLP76 were found to be highly phosphorylated in both cell types. Interestingly, PLC- γ 1 appears to be phosphorylated to slightly higher levels in Syk^{hi}/ZAP-70⁻ T cells as compared with control T cells. Moreover, a 36/38-kDa tyrosine-phosphorylated doublet that migrates with the same mobility as LAT was immunoprecipitated by both PLC- γ 1- and SLP76-specific antibodies (Fig. 3*C* and data not shown). Thus, in the presence of Syk and absence of ZAP-70,

³ N. Taylor, unpublished observations.

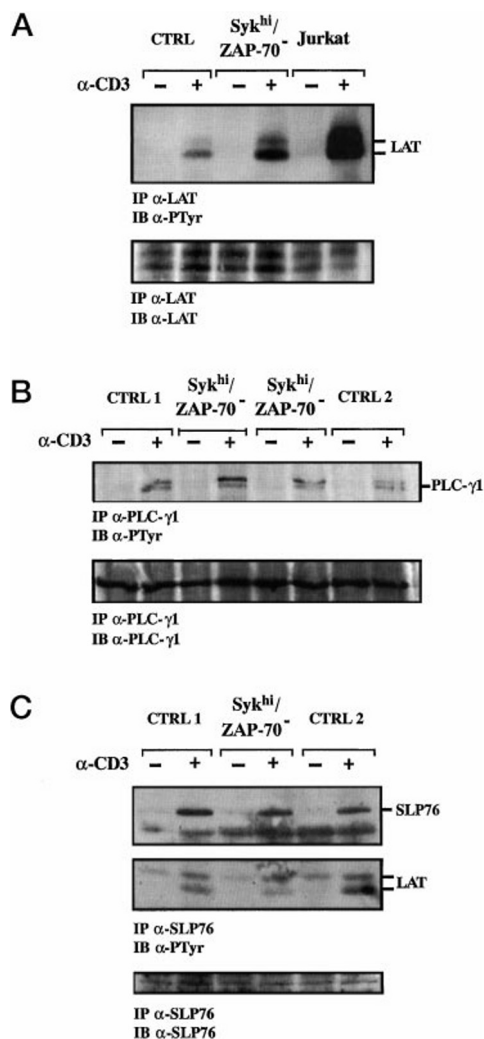


FIG. 3. LAT is tyrosine-phosphorylated and associates with PLC-γ1 and SLP76 in CD3-activated *Syk^{hi}/ZAP-70⁻* T cells. *A*, LAT was immunoprecipitated (IP) from control and *Syk^{hi}/ZAP-70⁻* T cells (1×10^7 cell equivalents) following either no stimulation (-) or stimulation with a cross-linked αCD3 mAb (+). Immunoprecipitates were immunoblotted (IB) with an α-Tyr(P) (α-P^{Tyr}) mAb to assess the phosphorylation status of LAT (upper panel) and then reprobbed with an αLAT pAb (bottom panel). *B*, lysates from nonstimulated (-) and CD3-stimulated (+) control and *Syk^{hi}/ZAP-70⁻* T cells were immunoprecipitated with αPLC-γ1 Ab, and the presence of tyrosine-phosphorylated PLC-γ1 was assessed by immunoblotting with an α-Tyr(P) mAb (upper panel). Blots were reprobbed with an αPLC-γ1 Ab (bottom panel). *C*, similarly, lysates were immunoprecipitated with an αSLP76 Ab, and the presence of tyrosine-phosphorylated proteins was monitored.

the activation of several important signaling intermediates is not adversely affected.

CD3-induced Calcium Mobilization Is Enhanced in *Syk^{hi}/ZAP-70⁻* T Cells—Next, we monitored the mobilization of intracellular calcium, another proximal signaling event. As illustrated in Fig. 4, CD3 cross-linking resulted in an increase in Ca^{2+} flux in *Syk^{hi}/ZAP-70⁻* T cells, as well as in T cells from 2 normal donors. Notably, Ca^{2+} mobilization was more rapid and higher in magnitude in *Syk^{hi}/ZAP-70⁻* T cells than in control T cells, despite equivalent levels of CD3 on the cell surface (Fig. 4A and data not shown). This difference in Ca^{2+} flux was also detected at lower concentrations of αCD3 mAb (1–5 μg/ml) (Fig. 4B). These observations indicate that early TCR-mediated signaling events are induced in *Syk^{hi}/ZAP-70⁻* T cells, albeit with some differences compared with control T cells expressing ZAP-70.

Syk^{hi}/ZAP-70⁻ T Cells Exhibit Decreased Erk, JNK, and p38

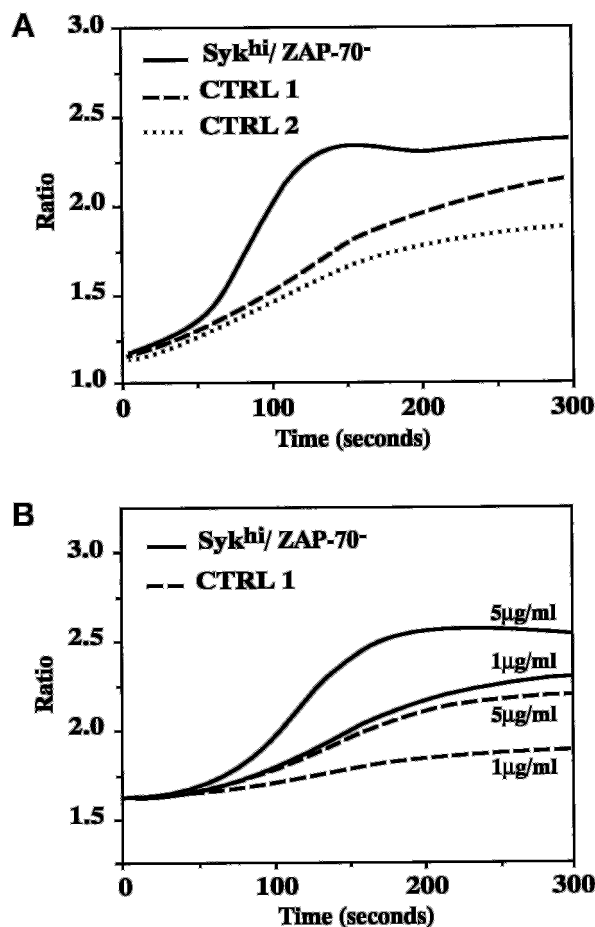


FIG. 4. Calcium mobilization is increased in CD3-cross-linked *Syk^{hi}/ZAP-70⁻* T cells. *Syk^{hi}/ZAP-70⁻* T cells and control T cells derived from two donors (CTRL 1 and CTRL 2) were loaded with the calcium-sensitive dye Indo-1 and analyzed for increases in $[Ca^{2+}]_i$ following stimulation with 10 μg/ml of the UCHT1 αCD3 mAb (*A*). Equivalent experiments were performed following stimulation with 1–5 μg/ml of the UCHT1 αCD3 mAb (*B*). Changes in $[Ca^{2+}]_i$ were monitored by fluorometry as a measure of the ratio of emission at 405/485 nm (ordinate axis) using a Photon Technologies spectrofluorometer for the indicated time period.

Activities—A growing body of evidence indicates that the Ras-Erk pathway is critical for the proliferation of distinct cell types (21). To assess whether activation of the MAPKs Erk1 and Erk2 was affected in *Syk^{hi}/ZAP-70⁻* T cells, their phosphorylation on residues Thr¹⁸³ and Tyr¹⁸⁵ and their kinase activity were analyzed. *Syk^{hi}/ZAP-70⁻* and control T cells were activated for 3 min with cross-linked αCD3 mAb, and the level of phosphorylated Erk proteins was assessed on immunoblots using a polyclonal Ab that specifically recognizes the dually phosphorylated form of Erk1 and Erk2. Although Erk1 and Erk2 proteins were phosphorylated in *Syk^{hi}/ZAP-70⁻* T cells in response to CD3 engagement, the level of phosphorylation was significantly lower than that detected in control T cells (Fig. 5A). Control blots showed that equivalent amounts of Erk2 were present in each lane (Fig. 5B). To further extend this observation, the ability of immunoprecipitated Erk1/Erk2 to phosphorylate an Erk substrate, MBP was assessed (Fig. 5C). Again, Erk-dependent kinase activity was clearly observed in CD3-activated *Syk^{hi}/ZAP-70⁻* T cells but at significantly lower levels than in control T cells. Thus, activation of Syk is associated with a decreased recruitment of the Ras-Erk cascade in *Syk^{hi}/ZAP-70⁻* T cells following CD3 engagement.

The MAPKs JNK and p38 are both stimulated by stress and have been implicated in apoptosis in certain cell systems (22,

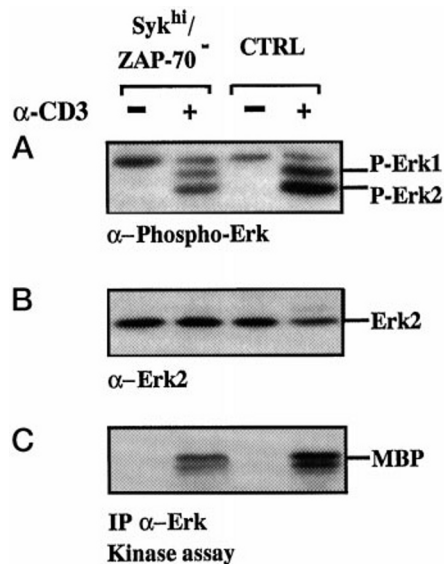


FIG. 5. Decreased Erk activation in *Syk^{hi}/ZAP-70⁻* T Cells stimulated via the TCR-CD3 complex. A, cell lysates (1×10^6 cell equivalents) were obtained from *Syk^{hi}/ZAP-70⁻* and control T cells (CTRL) that were either incubated in medium (-) or stimulated with an α CD3 mAb (+) for 3 min at 37 °C. Lysates were processed as described under "Experimental Procedures" and immunoblotted with a polyclonal Ab that recognizes the doubly phosphorylated forms of Erk1 and Erk2. B, the blot was stripped and reprobed with an α Erk2 mAb. C, Erk1 and Erk2 were immunoprecipitated from cell lysates (5×10^6 cell equivalents) using α Erk1/ α Erk2 pAbs, and kinase activity was determined by an immune complex kinase assay using MBP as a substrate.

23). However, JNK activation has also been observed following stimulation with various growth factors (24–26). Additionally, JNK is synergistically activated by costimulation of the CD3 and CD28 receptors (27). Therefore, we were interested in determining whether JNK and p38 were differentially activated in *Syk^{hi}/ZAP-70⁻* and control T cells. Cells were incubated in the presence or absence of either an α CD3 mAb or α CD3/ α CD28 mAbs for 5, 15, or 30 min, and the activation status of both JNK and p38 was monitored. In control T cells, the level of phosphorylated p54/p46 JNK proteins increased by 2.5–3.0-fold by 5 min after TCR engagement and returned to baseline levels by 30 min (Fig. 6A). In contrast, the phosphorylation of these proteins in *Syk^{hi}/ZAP-70⁻* T cells was lower, increasing by only 1.5-fold, and the kinetics of phosphorylation were longer, with a maximal response observed at 15 min (Fig. 6A). Similarly, 5 min following TCR costimulation, the level of phosphorylated p38 increased by 6.2-fold in control T cells and only 3.5-fold in *Syk^{hi}/ZAP-70⁻* T cells (Fig. 6B). It is important to note that anisomycin treatment (200 ng/ml) resulted in equivalent phosphorylation of JNK and p38 in control and *Syk^{hi}/ZAP-70⁻* T cells (data not shown), indicating that the latter cells had no intrinsic defect in JNK or p38 activities.

Collectively, these results demonstrate that although CD3 ligation results in the activation of several signaling intermediates and calcium mobilization in *Syk^{hi}/ZAP-70⁻* T cells, TCR-mediated recruitment of all three MAPK cascades is defective.

Defective TCR-induced IL-2 Secretion and Proliferation in *Syk^{hi}/ZAP-70⁻* T Cells—We next determined how the distinct TCR signaling cascade induced in *Syk^{hi}/ZAP-70⁻* T cells would affect the fate of these cells. As Erks, JNKs, and p38 play a role in the induction of IL-2 transcription (27), it was of interest to ascertain whether IL-2 secretion is compromised in these proliferating T cells derived from ZAP-70-deficient patients. IL-2 production was assessed 24 h following activation with immobilized α CD3 mAbs. IL-2 secretion was not detected in nonactivated *Syk^{hi}/ZAP-70⁻* T cells (<5 pg/ml), whereas a modest

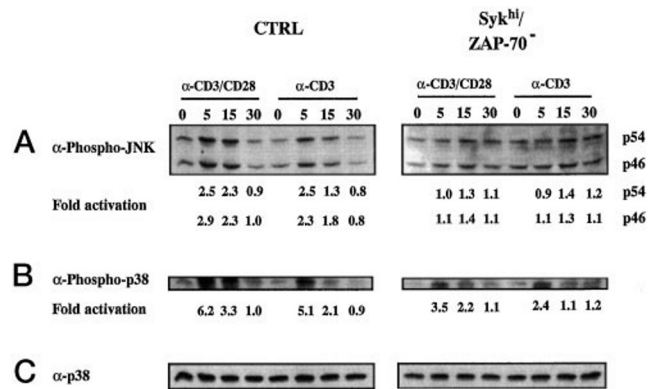


FIG. 6. JNK and p38 activation are decreased in *Syk^{hi}/ZAP-70⁻* T cells stimulated via the TCR-CD3 complex. Lysates (5.0×10^5 cell equivalents) were obtained from control (CTRL) and *Syk^{hi}/ZAP-70⁻* T cells that were stimulated with either α CD3 or α CD3/ α CD28 mAbs for 5, 15, or 30 min at 37 °C. A, the membrane was immunoblotted with Abs that recognizes the phosphorylated p54/p46 isoforms of JNK. B, lysates from the same experiment were immunoblotted with a polyclonal Ab that recognizes the phosphorylated form of p38 (α -phospho-p38). C, the blot was stripped and reprobed with an α -p38 Ab to ensure equivalent protein loading in each lane. The fold increase in JNK and p38 activation relative to unstimulated cells is indicated.

TABLE I
CD3-induced IL-2 secretion

Cells were stimulated with immobilized anti-CD3 mAb, and IL-2 secretion was measured 24 h later by enzyme-linked immunosorbent assay. The results represent the mean \pm S.D. of triplicate samples. CTRL, control.

Anti-CD3	IL-2	
	-	+
	pg/ml	
<i>Syk^{hi}/ZAP-70⁻</i>	<5	167 \pm 12
CTRL 1	<5	2101 \pm 307
CTRL 2	<5	909 \pm 259

level was induced by α CD3-activation (167 pg/ml). Nevertheless, these levels were significantly lower than those detected in equivalently treated control T cells (909–2101 pg/ml) (Table I). These results indicate that IL-2 secretion is compromised but not absent in CD3-stimulated T cells that exhibit defective MAPK activation.

Although *Syk^{hi}/ZAP-70⁻* T cells clearly proliferated in response to the extensive mixture of mitogenic agents present in the culture media, it was important to determine their proliferative capacity following activation of the TCR alone. Thus, [3 H]thymidine incorporation was measured 3 days after activation with either immobilized α CD3 mAb, α CD3/ α CD28 mAbs, a combination of IL-2 and α CD3 mAbs, or exogenous IL-2 alone. Unlike freshly obtained ZAP-70^{-/-} T cells, which did not proliferate following CD3 or CD3/ α CD28 engagement, the corresponding *Syk^{hi}/ZAP-70⁻* T cells responded to these stimuli (Fig. 7 and data not shown). However, proliferation of the latter cells was markedly decreased compared with equivalently treated control T cells ($p = 0.003$). The decreased CD3-induced proliferation in *Syk^{hi}/ZAP-70⁻* T cells was not solely due to decreased IL-2 secretion as addition of exogenous IL-2 did not alleviate this difference. Nevertheless, *Syk^{hi}/ZAP-70⁻* T cells were able to respond to IL-2 alone with a similar level of proliferation as control T cells (not shown). Altogether, these results indicate that in the context of T cells expressing Syk but not ZAP-70, a 2–3-fold decrease in the activation of all three MAPK cascades is associated with a profound defect in CD3-mediated IL-2 secretion and proliferation.

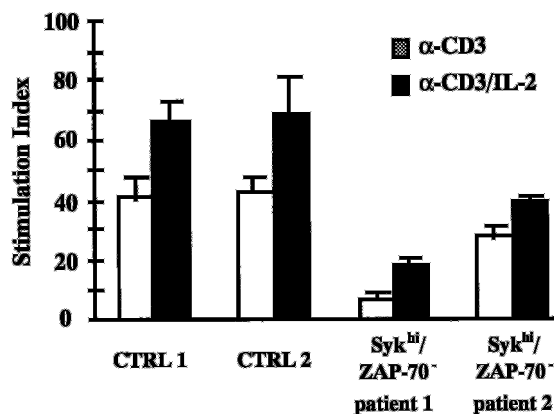


FIG. 7. Defective TCR-CD3-induced proliferation in *Syk*^{hi}/*ZAP-70*⁻ cells. *Syk*^{hi}/*ZAP-70*⁻ T cells derived from both *ZAP-70*-deficient patients and control T cells were stimulated with either immobilized α CD3 mAb (1 μ g/ml) or a combination of exogenous IL-2 (100 U/ml) and α CD3 mAbs. After 3 days of culture, cells were pulsed with 1 μ Ci of [³H]thymidine and harvested 18 h later. Values shown are the mean \pm SD of data obtained in one of three representative experiments. The stimulation index was determined as described under "Experimental Procedures."

DISCUSSION

Here, we demonstrate that the *in vitro* proliferation of primary CD4⁺ α β T cells from *ZAP-70*-deficient patients consistently results in the expansion of cells expressing high levels of Syk. Thus, these cells provide a unique model for assessing TCR-induced biological responses associated with the activation of Syk in a nontransformed T cell context. Although the endogenous levels of Syk and *ZAP-70* cannot be easily compared, it is important to note that the high level of Syk observed in the primary T cells utilized here is equivalent to that detected in the transformed Jurkat T cell line. Stimulation of primary *Syk*^{hi}/*ZAP-70*⁻ T cells via the TCR-CD3 complex resulted in the phosphorylation of Syk suggesting that Syk may substitute for *ZAP-70* in transducing extracellular signals. Indeed, the consistent *in vitro* outgrowth of *ZAP-70*-deficient T cells with high Syk levels likely reflects their acquisition of a significant response to exogenous stimulus.

Accordingly, CD3-induced responses, including tyrosine phosphorylation of cellular substrates that are defective in freshly obtained *ZAP-70*-deficient T cells expressing only low levels of Syk (this work),² were observed in these *Syk*^{hi}/*ZAP-70*⁻ T cells. Importantly, LAT, which associates with the CD4 itself and links the activation of proximal kinases with downstream events (20, 28), was phosphorylated in CD3-engaged *Syk*^{hi}/*ZAP-70*⁻ T cells. Finally, in *Syk*^{hi}/*ZAP-70*⁻ T cells, CD3 stimulation induced the interaction of PLC- γ 1 and SLP76 with a 36/38 *K_d* doublet, which is presumably LAT. Nevertheless, we cannot exclude the possibility that the association of LAT with other adapter proteins, such as Grb2, is defective in *Syk*^{hi}/*ZAP-70*⁻ T cells. The interaction of LAT with Grb2 or Grb2-like adapters, such as Grap or Gads, is thought to be crucial for the recruitment of SOS, a guanine-nucleotide-exchange factor that converts the GDP-bound form of Ras into an active GTP-bound form (29). However, because Grb2 is expressed at very low levels in primary T cells (in contrast with Jurkat T cells, in which it is expressed at high levels), its association with LAT could not be monitored. Further work will establish whether the association of LAT with other adapter proteins such as Grap or Gads may differ in T cells, in which there is an activation of Syk but not *ZAP-70*. Indeed, it is clear that differences exist in T cells in which one or another of these kinases is phosphorylated. Specifically, whereas the ζ chain of the TCR is phosphorylated following TCR engagement of T cells express-

ing *ZAP-70*, ζ chain phosphorylation is severely attenuated in a *ZAP-70*-deficient thymocyte line expressing high levels of Syk (8), a Jurkat clone in which Syk was transiently expressed (15), and the *Syk*^{hi}/*ZAP-70*⁻ T cells assessed here.⁴ As the interaction of *ZAP-70* with the phosphorylated ζ subunit is required for appropriate downstream signaling, these data point to a crucial difference between *ZAP-70* and Syk. Thus, the propagation of a TCR signaling cascade in T cells expressing either Syk or *ZAP-70* may already differ at the level of the TCR itself.

In this regard, it is important to note that although many signaling intermediates appeared to be equivalently activated in *Syk*^{hi}/*ZAP-70*⁻ and control T cells, proximal signaling in these two cell types was not identical; the CD3-mediated increase in intracellular calcium in *Syk*^{hi}/*ZAP-70*⁻ T cells was more rapid and significantly higher in magnitude than that detected in equivalently treated control T cells. This increase may indeed be due to the higher level of PLC- γ 1 phosphorylation observed in *Syk*^{hi}/*ZAP-70*⁻ T cells. Interestingly, in the a similar phenomenon has been reported in murine *ZAP-70*-deficient thymocytes containing transgenic Syk, in which CD3 cross-linking resulted in increased calcium flux relative to wild-type thymocytes (30). Together, these data strongly support our hypothesis that the activation of Syk is responsible for the CD3-induced phosphorylation of signaling intermediates and increased calcium mobilization observed in the *Syk*^{hi}/*ZAP-70*⁻ T cells described here.

Recent work suggests that different levels of calcium flux can lead to the propagation of distinct downstream signals. In B cells, the amplitude and duration of dynamic calcium signals has been shown to differentially activate the transcription regulators NF κ B and NFAT, as well as the MAPKs Erk and JNK (31). We now extend this observation to show that an increased calcium flux is linked with a defective induction of all three MAPK cascades in *Syk*^{hi}/*ZAP-70*⁻ T cells. This is the first demonstration that stimulation of Syk is associated with a distinct TCR signaling cascade involving decreased MAPK activation. It is interesting to note that whereas we observed a defective JNK activation in *Syk*^{hi}/*ZAP-70*⁻ T cells, Jacinto *et al.* (32) found that Syk enhances the CD3/CD28-induced activation of JNK in the Jurkat T cell line. However, unlike the experiments presented here, the aforementioned study was performed in a transformed cell line that expresses *ZAP-70*. Furthermore, Latour *et al.* (14) found that the introduction of Syk into a *ZAP-70*-expressing murine T cell hybridoma enhanced T cell responsiveness. Thus, the presence of *ZAP-70* appears to modulate the role of Syk in TCR signaling.

The Erk, JNK, and p38 MAPK pathways have all been implicated in T cell mitogenesis and IL-2 secretion (27, 33, 34). Indeed, inhibition of any of these three pathways has been found to result in decreased IL-2 secretion (33, 34). It was therefore not surprising to observe a decreased level of CD3-induced IL-2 secretion in the *Syk*^{hi}/*ZAP-70*⁻ T cells described here. The importance of IL-2 production in T cell mitogenesis is underscored by the finding that CD3-induced proliferation is severely impaired in murine and human T cells that cannot secrete IL-2 as a result of mutations in the IL-2 gene (35–37). However, in contrast with these IL-2-deficient cells in which CD3-induced proliferation increases to normal levels in the presence of recombinant IL-2, the profound proliferation defect in CD3-induced *Syk*^{hi}/*ZAP-70*⁻ T cells could not be alleviated by the addition of exogenous IL-2. In this regard, it is notable that IL-2, although required for optimal T cell growth and survival, sensitizes cells to TCR activation-induced cell death (38, 39). Indeed, we find a significantly higher level of activa-

⁴ N. Noraz and N. Taylor, unpublished observations.

tion-induced cell death in *Syk^{hi}/ZAP-70⁻* T cells stimulated by an anti-CD3 mAb in the presence of IL-2 than in equivalently treated control T cells.⁵ Thus, it is likely that the balance between apoptosis and growth is weighted toward the former in these *Syk^{hi}/ZAP-70⁻* T cells, accounting for the decreased level of TCR-induced proliferation. Importantly, our recent finding that CD3-mediated proliferation of *Syk^{hi}/ZAP-70⁻* T cells can be corrected by introduction of the wild-type ZAP-70 gene demonstrates that in these cells, all effector molecules required for T cell activation, with the exception of ZAP-70, are functional.⁶

The down-regulation of Syk expression between the double negative and double positive stage of thymocyte development appears to be more pronounced in mice than in humans (40) and may explain why, in the absence of ZAP-70, CD4⁺ T cells develop in the latter but not in the former. It is important to note that in contrast with Syk, ZAP-70 levels do not change during T cell ontogeny. It is likely that the mature CD4⁺ T cells in the periphery of ZAP-70^{-/-} patients cannot respond to mitogens because Syk is further down-regulated (Ref. 7 and this work). In this regard, it is notable that Syk is apparently not down-regulated in all human T cells; a rare subpopulation of normal $\alpha\beta$ T cells expressing high levels of Syk has been discussed in a recent review (40). Although these cells have not been characterized and their physiological relevance is not clear, it is tempting to speculate that this subpopulation also exists in ZAP-70-deficient patients and represents the polyclonal population that proliferated in response to the extensive mixture of mitogenic agents used in this study. As the *Syk^{hi}/ZAP-70⁻* T cells described here demonstrated a distinct response to TCR engagement, it will be important to determine whether there are functional differences between normal ZAP-70⁺ T cells expressing high Syk and the vast majority of ZAP-70⁺ T cells that express low or undetectable levels of Syk. Further work will help to elucidate whether the fate of normal thymocytes and T cells is modulated by the relative activation of ZAP-70 and Syk.

Acknowledgments—We are grateful to P. Jourdan, V. Richard, and S. Simic for their assistance. We also thank C. Hivroz, K. Weinberg, and E. Gelfand for sharing information regarding three other ZAP-70-deficient patients; A. Weiss for his input during initial stages of this work; and R. Wange, G. Koretzky, and A. Singer for helpful discussions. We are indebted to M. Sitbon for his constructive comments throughout the course of this study.

REFERENCES

1. Chan, A. C., Iwashima, M., Turck, C. W., and Weiss, A. (1992) *Cell* **71**, 649–662
2. Arpaia, E., Shahar, M., Dadi, H., Cohen, A., and Roifman, C. M. (1994) *Cell* **76**, 947–958
3. Chan, A. C., Kadlecsek, T. A., Elder, M. E., Filipovich, A. H., Kuo, W. L.,

- Iwashima, M., Parslow, T. G., and Weiss, A. (1994) *Science* **264**, 1599–1601
4. Elder, M. E., Lin, D., Clever, J., Chan, A. C., Hope, T. J., Weiss, A., and Parslow, T. G. (1994) *Science* **264**, 1596–1599
5. Negishi, I., Motoyama, N., Nakayama, K., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A. C., and Loh, D. Y. (1995) *Nature* **376**, 435–438
6. Wiest, D. L., Ashe, J. M., Howcroft, T. K., Lee, H. M., Kemper, D. M., Negishi, I., Singer, D. S., Singer, A., and Abe, R. (1997) *Immunity* **6**, 663–671
7. Chan, A. C., van Oers, N. S. C., Tran, A., Turka, L., Law, C. L., Ryan, J. C., Clark, E. A., and Weiss, A. (1994) *J. Immunol.* **152**, 4758–4766
8. Gelfand, E. W., Weinberg, K., Mazer, B. D., Kadlecsek, T. A., and Weiss, A. (1995) *J. Exp. Med.* **182**, 1057–1065
9. Latour, S., Chow, L., and Veillette, A. (1996) *J. Biol. Chem.* **271**, 22782–22790
10. Kolanus, W., Romeo, C., and Seed, B. (1993) *Cell* **74**, 171–183
11. Couture, C., Baier, G., Altman, A., and Mustelin, T. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 5301–5305
12. Taylor, N., Jahn, T., Smith, S., Lamkin, T., Uribe, L., Liu, Y., Durden, D. L., and Weinberg, K. (1997) *Blood* **89**, 388–396
13. Chu, D. H., Spits, H., Peyron, J. F., Rowley, R. B., Bolen, J. B., and Weiss, A. (1996) *EMBO J.* **15**, 6251–6261
14. Latour, S., Fournel, M., and Veillette, A. (1997) *Mol. Cell. Biol.* **17**, 4434–4441
15. Williams, B. L., Schreiber, K. L., Zhang, W., Wange, R. L., Samelson, L. E., Leibson, P. J., and Abraham, R. T. (1998) *Mol. Cell. Biol.* **18**, 1388–1399
16. Yssel, H., de Vries, J. E., Koken, M., Van Blitterswijk, W., and Spits, H. (1984) *J. Immunol. Methods* **72**, 219–227
17. Spits, H., Yssel, H., Terhorst, C., and de Vries, J. E. (1982) *J. Immunol.* **128**, 95–99
18. Taylor, N., Uribe, L., Smith, S., Jahn, T., Kohn, D. B., and Weinberg, K. (1996) *Blood* **87**, 3103–3107
19. Matsuda, S., Suzuki-Fujimoto, T., Minowa, A., Ueno, H., Katamura, K., and Koyasu, S. (1999) *J. Biol. Chem.* **274**, 34515–34518
20. Zhang, W., Sloan, L. J., Kitchen, J., Triple, R. P., and Samelson, L. E. (1998) *Cell* **92**, 83–92
21. Cantrell, D. (1996) *Annu. Rev. Immunol.* **14**, 259–274
22. Wilson, D. J., Fortner, K. A., Lynch, D. H., Mattingly, R. R., Macara, I. G., Posada, J. A., and Budd, R. C. (1996) *Eur. J. Immunol.* **26**, 989–994
23. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) *Science* **270**, 1326–1331
24. Minden, A., Lin, A., McMahon, M., Lange, C. C., Derijard, B., Davis, R. J., Johnson, G. L., and Karin, M. (1994) *Science* **266**, 1719–1723
25. Nagata, K., Nakamura, T., Kitamura, F., Kuramochi, S., Taki, S., Campbell, K. S., and Karasuyama, H. (1997) *Immunity* **7**, 559–570
26. Timokhina, I., Kissel, H., Stella, G., and Besmer, P. (1998) *EMBO J.* **17**, 6250–6262
27. Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben-Neriah, Y. (1994) *Cell* **77**, 727–736
28. Bosselut, R., Zhang, W., Ashe, J. M., Kopacz, J. L., Samelson, L. E., and Singer, A. (1999) *J. Exp. Med.* **190**, 1517–1526
29. Izquierdo Pastor, M., Reif, K., and Cantrell, D. (1995) *Immunol. Today* **16**, 159–164
30. Gong, Q., White, L., Johnson, R., White, M., Negishi, I., Thomas, M., and Chan, A. C. (1997) *Immunity* **7**, 369–377
31. Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C., and Healy, J. I. (1997) *Nature* **386**, 855–858
32. Jacinto, E., Werlen, G., and Karin, M. (1998) *Immunity* **8**, 31–41
33. Matsuda, S., Moriguchi, T., Koyasu, S., and Nishida, E. (1998) *J. Biol. Chem.* **273**, 12378–12382
34. Li, Y. Q., Hii, C. S., Der, C. J., and Ferrante, A. (1999) *Immunology* **96**, 524–528
35. Schorle, H., Holtschke, T., Hunig, T., Schimpl, A., and Horak, I. (1991) *Nature* **352**, 621–624
36. Razi-Wolf, Z., Hollander, G. A., and Reiser, H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 2903–2908
37. Pahwa, R., Chatila, T., Pahwa, S., Paradise, C., Day, N. K., Geha, R., Schwartz, S. A., Slade, H., Oyaizu, N., and Good, R. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 5069–5073
38. Van Parijs, L., Biuckians, A., Ibragimov, A., Alt, F. W., Willerford, D. M., and Abbas, A. K. (1997) *J. Immunol.* **158**, 3738–3745
39. Refaeli, Y., Van Parijs, L., London, C. A., Tschopp, J., and Abbas, A. K. (1998) *Immunity* **8**, 615–623
40. Chu, D. H., Morita, C. T., and Weiss, A. (1998) *Immunol. Rev.* **165**, 167–180

⁵ N. Noraz, unpublished observations.

⁶ Steinberg, M., and Noraz, N. (2000) *Gene Ther.*, in press.