

# Defective Expression of p56lck in an Infant with Severe Combined Immunodeficiency

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## Abstract

Severe combined immune deficiency (SCID) is a heterogeneous disorder characterized by profound defects in cellular and humoral immunity. We report here an infant with clinical and laboratory features of SCID and selective CD4 lymphopenia and lack of CD28 expression on CD8<sup>+</sup> T cells. T cells from this patient showed poor blastogenic responses to various mitogens and IL-2. Other T cell antigen receptor–induced responses, including upregulation of CD69, were similarly inhibited. However, more proximal T cell antigen receptor signaling events, such as anti-CD3 induced protein tyrosine phosphorylation, phosphorylation of mitogen-associated protein kinase, and calcium mobilization were intact. Although p59fyn and ZAP-70 protein tyrosine kinases were expressed at normal levels, a marked decrease in the level of p56lck was noted. Furthermore, this decrease was associated with the presence of an alternatively spliced *lck* transcript lacking the exon 7 kinase encoding domain. These data suggest that a deficiency in p56lck expression can produce a SCID phenotype in humans. (*J. Clin. Invest.* 102: 421–429.) Key words: tyrosine kinases • signal transduction • T lymphocytes • *lck* • severe combined immune deficiency

## Introduction

Severe combined immune deficiency (SCID)<sup>1</sup> represents a spectrum of congenital disorders characterized by profound defects in both humoral and cellular immunity. Affected infants commonly present in the first year of life with failure to thrive, opportunistic infections, and low or absent immunoglobulin levels. The most common form of X-linked SCID results from mutations in the common cytokine receptor gamma chain, which is shared by the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors (1). The importance of these receptors in T and B cell activation and T cell development has been well documented (1). Additionally, autosomal recessive forms of SCID

have been shown to be caused by defects in T cell receptor subunits or signaling molecules including CD3 (2, 3), IL-2/NFAT-1 (4), JAK3 (5, 6), and ZAP-70 (7–9).

The T cell antigen receptor (TCR) plays a crucial role in thymocyte differentiation and T cell activation (10, 11). After antigen binding to the TCR, and in concert with engagement of other coreceptors and their associated ligands (e.g., CD4 and MHC II, CD28 and B7, CD8 and MHC I), signal transduction cascades are activated. The earliest measurable biochemical event is the activation of protein tyrosine kinases (PTKs), resulting in the phosphorylation of multiple cellular substrates. At least three PTKs are known to be involved in TCR signaling at the level of the receptor, including p59fyn, p56lck, and ZAP-70 (8, 12). Recently, individuals with mutations in the gene coding for the ZAP-70 protein have been identified and these patients present with an absence of peripheral CD8<sup>+</sup> T cells and normal to high levels of peripheral CD4<sup>+</sup> T cells which are unable to signal through the TCR (7–9). SCID-like phenotypes have also been observed in mice rendered p56lck or p59fyn deficient by homologous recombination (13–15). Thymocytes from *fyn* knockout mice do not signal normally through the TCR and mice lacking p56lck as well as transgenic mice expressing dominant negative mutations of p56lck demonstrate severe T cell developmental defects (13–16). Moreover, T cell development is blocked at the CD4<sup>+</sup>/CD8<sup>−</sup> thymocyte stage in mice deficient for both p56lck and p59fyn (17, 18). There have been no reported cases of defective p59fyn or p56lck associated with congenital immune deficiencies in humans.

In this report, we describe a unique form of SCID in a patient with selective CD4 lymphopenia and markedly reduced production of the p56lck protein. The decreased expression of p56lck in this patient is likely the result of an alteration in the splicing pattern of exon 7 of the *lck* mRNA.

## Methods

**Immunological reagents.** Antibodies reacting with T cell (CD69 [Leu23a], CD4 [Leu3a], CD3 [Leu4]), B cell (CD20 [Leu16]), and NK cell markers (CD16/56 [Leu11c/Leu19]) were purchased from Becton Dickinson (Mountain View, CA). Other commercial antibodies include FITC-conjugated goat anti-mouse secondary antibody (Organon Teknika-Cappel, Durham, NC), polyclonal antiphosphotyrosine antibody (4G10: Upstate Biotechnology, Inc., Lake Placid, NY), polyclonal rabbit anti-rat ERK-2 (Upstate Biotechnology, Inc.), goat anti-mouse Ig (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Bio-Rad Laboratories, Hercules, CA). p56lck and p59fyn antibodies were generated by immunizing rabbits with peptides to unique sequences within these molecules (*lck* amino acid residues 39–64 and *fyn* residues 29–48), and affinity purifying the antibodies over a column containing the peptides coupled to cyanogen bromide activated Sepharose beads. Polyclonal anti-ZAP-70 and anti-*lck* antibodies (see Fig. 2 B) were generous gifts from A. Weiss (University of California, San Francisco, CA) and B. Sefton (Salk In-

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1. Abbreviations used in this paper: [Ca<sup>2+</sup>], intracellular free calcium concentration; PKC, protein kinase C; PTK, protein tyrosine kinase; RT, reverse transcription; SCID, severe combined immune deficiency; TCR, T cell antigen receptor.

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stitute, La Jolla, CA), respectively. OKT3 was purchased from PharMingen (San Diego, CA). The following commercial reagents were also employed: phytohemagglutinin (Murex Diagnostics, Saline, MI), pokeweed mitogen (GIBCO BRL, Gaithersburg, MD), concanavalin A (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), and IL-2 (Cetus Corp., Emeryville, CA).

**Cells.** PBMC were isolated from healthy donors and the patient by density gradient centrifugation (Ficoll-Hypaque). Adherent cells were removed by adherence to plastic dishes for 2 h at 37°C in 5% CO<sub>2</sub>. For controls in some of the experiments, normal PBMC were enriched for CD8<sup>+</sup> T lymphocytes by negative selection using anti-CD4-conjugated BioMag magnetic beads (PerSeptive Diagnostics, Cambridge, MA). Flow cytometric analysis of negatively selected control CD8<sup>+</sup> PBMC demonstrated a CD4:CD8 ratio of 0.2–0.5, similar to that observed in the mononuclear fraction of the patient cells. Jurkat (clone E6-1; American Type Culture Collection [ATCC], Rockville, MD) and JCaM.1 cells, derived from the parent Jurkat cell line as previously described (19), were maintained in RPMI 1640 supplemented with 10% FCS, penicillin (1,000 U/ml), streptomycin (1,000 U/ml), and glutamine (20 mM).

**Flow cytometry.** For determination of cell surface antigens, Ficoll-Hypaque-separated mononuclear cells were incubated with primary antibodies, washed twice, then stained with a secondary fluorescein or phycoerythrin-labeled goat anti-mouse antibody. Fluorescence was examined using an EPICS 753 (Coulter Immunology, Hialeah, FL).

**Analysis of intracellular free calcium concentration.** Intracellular free calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) was determined by flow microfluorimetry (EPICS 753) as previously described (20). Briefly, mononuclear cells were washed, resuspended at 5 × 10<sup>6</sup> cells/ml in Indo A buffer (IMDM, 10 mM Hepes, pH 7.0), and loaded with the calcium-sensitive fluorescent dye Indo-1 AM (Molecular Probes, Inc., Eugene, OR). After 30 min at 37°C, an equal amount of Indo B buffer (IMDM, 10 mM Hepes, 5% FCS, pH 7.4) was added and cells were incubated for an additional 30 min at 37°C. Finally, cells were washed in Indo C (IMDM, 5% FCS, 10 μg/ml DNase), resuspended at 2 × 10<sup>6</sup>/ml, and kept at room temperature until 15 min before analysis when they were warmed to 37°C. Analyses of [Ca<sup>2+</sup>]<sub>i</sub> were conducted at 37°C, and data are displayed as relative [Ca<sup>2+</sup>]<sub>i</sub> versus time.

**Cell stimulation and Western blotting.** Cells (10<sup>7</sup>/ml) were stimulated in serum-free RPMI 1640 at 37°C with OKT3 (40 μg/ml) for 1 min, followed by addition of secondary cross-linking goat anti-mouse Ig (1:100) for 1–30 min as indicated. Reactions were terminated by addition of ice cold PBS, and then lysed in 1% NP-40 detergent buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 7.4) containing protease (50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin A, 1 mM polymethylsulfonyl fluoride), and phosphatase (400 nM sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate) inhibitors. After incubation on ice for 15 min, lysates representing one million cell equivalents per time point were clarified by high speed centrifugation (14,000 rpm), reduced in 2× SDS sample buffer, boiled for 5 min, separated by 10% SDS-PAGE, and transferred electrophoretically to nitrocellulose. Membranes were blocked overnight with 3% BSA in Tris-buffered saline-0.1% Tween and primary antibodies were added for 4 h at 4°C. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) or BCIP/NBT (Promega Corp., Madison, WI) following the manufacturer's instructions.

**Preparation of RNA, DNA, and reverse transcription PCR.** RNA and DNA were extracted from PBMC using Triagent (Sigma Chemical Co., St. Louis, MI), as described by the manufacturer. For reverse transcription (RT)-PCR reactions, *lck* cDNA was amplified by RT using 1 μg of purified RNA, oligo-dT as a primer and AMV reverse transcriptase in a buffer supplied by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, IN). PCR primers (1F, 5'-CGCCTGGACCATGTGAAT-3'; 415F, 5'-CTGGAGCCGAACCCTGG-3'; 754F, 5'-GAGGACGAGTGGGAGGTTCC-3'; 756R, 5'-CTCCCACCACGGCTTCTG-3'; 1482R, 5'-CAGCA-

CACTGCGCAGGTA-3'; 1581R, 5'-GCTCTAGATCAAGGCTG-AGGCTGGTACT-3') were derived from the published sequence of the human *lck* gene (21). Splicing sites surrounding exon 7 were examined by amplifying genomic DNA with primers derived from the published sequence and located within exons 6 and 8 (551F, 5'-GTAATCTGGACAACGGTGGC; 816R, 3'-CTTCAGGCTCTTACC GC). PCR reactions were performed using 1 μl of RT reaction as template, gene specific primers (1 μM final concentration), and Taq DNA polymerase (Boehringer Mannheim Biochemicals).

The resulting PCR products were gel purified (Bio 101, La Jolla, CA) and sequenced with an ABI Model 373 Automated DNA Sequencer (PE-Applied Biosystems, Foster City, CA) at the University of Iowa DNA Core Facility (Iowa City, IA).

**Blastogenic assays.** PBMC were purified by Ficoll-Hypaque and cultured, in triplicate, in microtiter wells, at 4 × 10<sup>5</sup> cells per well, either with medium alone or with the indicated stimuli. For mitogenic stimulation (Con A, PHA, PWM, anti-CD3), cells were cultured for 3 d in a humidified 37°C incubator. For allogeneic stimulations, cells were cultured for 6 d in the presence of 5 × 10<sup>4</sup>-irradiated EBV-transformed cells. On day 3 or 6, the wells were pulsed with 1 μCi of [<sup>3</sup>H]thymidine, and after 4 h [<sup>3</sup>H]incorporation was assessed in a β scintillation counter (22).

**Analysis of CD69 expression.** PBMC were cultured at 4 × 10<sup>6</sup> cells per well in 24-well culture plates for 24 h. Wells were supplemented with PMA and ionomycin, culture medium alone, or immobilized anti-CD3. For immobilized anti-CD3, wells were coated with 0.5 ml of purified anti-CD3 (PharMingen) at 3 μg/ml for 4 h at 37°C after which time the wells were washed and PBMC were added. After a 24-h stimulation, cells were stained and analyzed by flow cytometry using an Epics 753 instrument.

## Results

**Clinical course.** The patient was a male infant of a nonconsanguineous union who was noted to have loose stools and poor weight gain at 1 mo of age. He was initially hospitalized at 2 mo of age for dehydration, failure to thrive, and sepsis. Physical examination at that time was remarkable for the presence of oral candidiasis and a weight below the fifth percentile. Multiple cultures were obtained and rotavirus was isolated from the stool, *Enterobacter cloacae* from the blood, and cytomegalovirus from the urine and intestinal biopsies. As part of an immune assessment, lymphopenia and hypogammaglobulinemia were noted (Tables I and II). Tests for HIV in the patient (Western blotting, PCR, and serologies) and mother (serologies) were negative on multiple occasions, and other causes of SCID, including deficiencies of adenosine deaminase and purine nucleoside phosphorylase, were excluded. Cytogenetic analysis of peripheral lymphocytes revealed a normal XY karyotype without evidence of maternal engraftment. The pa-

Table I. Serum Immunoglobulins

Age	IgG (252–708)	IgA (22–129)	IgM (33–155)	IgE (10–180)
mo				
1	105	6	5	—
3	309*	2	16	—
8	736*	—	< 5	—
16	636*	—	< 5	—
28	636*	22	6	2

\*On IVIG.

Table II. Immunophenotype

Age (mo)	2	8	16	24	Normal (12–18 m)
Total lymph count	1580	1890	1380	780	4000–10500
CD antigen					
CD3	760 (48%)	1191 (63%)	1035 (75%)	507 (85%)	2000–7035 (55–71%)
CD4	330 (21%)	170 (9%)	124 (9%)	172 (22%)	1520–4830 (38–46%)
CD8	430 (27%)	945 (50%)	718 (52%)	491 (63%)	640–2730 (16–26%)
CD4:CD8	0.8	0.2	0.2	0.3	1.5–2.0
CD16 <sup>+</sup> /56 <sup>+</sup> /3 <sup>-</sup>	80 (5%)	57 (3%)	69 (5%)	39 (5%)	270–1100 (7–21%)
CD20	710 (45%)	624 (33%)	221 (16%)	296 (38%)	800–3600 (20–32%)
CD8 <sup>+</sup> /28 <sup>+</sup>	—	—	2%	7%	80%
CD8 <sup>+</sup> /28 <sup>-</sup>	—	—	98%	93%	20%
CD4 <sup>+</sup> /28 <sup>+</sup>	—	—	83%	85%	90%
CD4 <sup>+</sup> /28 <sup>-</sup>	—	—	17%	15%	10%

tient was begun on intravenous immunoglobulin and remained hospitalized for nearly 8 mo secondary to recurrent infections and profuse diarrhea. He continued to have significant infectious and gastrointestinal problems, and remained dependent on total parenteral nutrition. In an effort to increase the patient's CD4<sup>+</sup> T cell count, a 2-mo trial of IL-2 was begun when the patient was 26-mo-old, but this elicited neither a clinical response nor an improvement in lymphocyte numbers. The patient has since undergone an allogeneic bone marrow transplant (at 32 mo of age) from a matched unrelated donor.

*SCID phenotype is associated with a selective CD4 lymphopenia and an inverted CD4/CD8 ratio.* Expression of lymphoid surface molecules was monitored over a 28-mo period in this patient (Table II). The absolute lymphocyte count, while fluctuating, was always decreased relative to age matched controls (780–1,890 versus 4,000–10,000). Between 8 and 24 mo of age, analysis of T lymphocyte subsets revealed a relative increase in the percentage of CD8<sup>+</sup> T lymphocytes (50–63%), which expressed both TCR $\alpha$  and TCR $\beta$  chains, while the absolute count was decreased or in the low normal range (490–945). In contrast, the relative percentage of CD4<sup>+</sup> T cells was consistently low (9–22%), with a marked decrease in absolute counts that never exceeded 350/mm<sup>3</sup>. The CD4/CD8 ratio was thus inverted, ranging between 0.2 and 0.3 from 8–24 mo of age. Further immunophenotyping also excluded other causes of CD4 lymphopenia, including lack of HLA-DR expression or CD40 ligand deficiency (data not shown). B (CD19) and NK (CD16<sup>+</sup>/56<sup>+</sup>/3<sup>-</sup>) cell numbers were variable and while the relative percentages were in the normal range, the absolute counts were in the low to low-normal range. Expression of accessory molecules was also examined within the CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations. CD28, a T cell costimulatory molecule, was nearly absent on the CD8<sup>+</sup> population, accounting for only 2–7% of CD8<sup>+</sup> T cells, while 70–90% of control CD8<sup>+</sup> cells expressed CD28. However, CD28 expression on the patient's CD4<sup>+</sup> population was within normal limits, with 85% of CD4<sup>+</sup> T cells coexpressing this molecule.

*CD3-induced tyrosine phosphorylation of cellular substrates.* Tyrosine phosphorylation of multiple cellular proteins, the result of PTK activation, is one of the earliest biochemical events induced after TCR engagement (23–25). To determine the overall integrity of PTKs in patient T cells, freshly isolated

cells were stimulated with OKT3 plus secondary goat anti-mouse antibodies, and protein tyrosine phosphorylation was assessed by immunoblotting cellular lysates with an antiphosphotyrosine monoclonal antibody. As shown in Fig. 1, proteins with molecular masses of 36, 40, and 80 kD were tyrosine phosphorylated in both control and patient T cells. The only obvious difference between the patient and the control was a sustained phosphorylation of an 80-kD substrate. Thus, unlike patients with ZAP-70 deficiency where tyrosine phosphorylation of cellular substrates is severely impaired upon TCR cross-linking (9), the patient described here demonstrated a relatively normal pattern of tyrosine phosphorylation.

*Decreased expression of the p56lck tyrosine kinase in pa-*

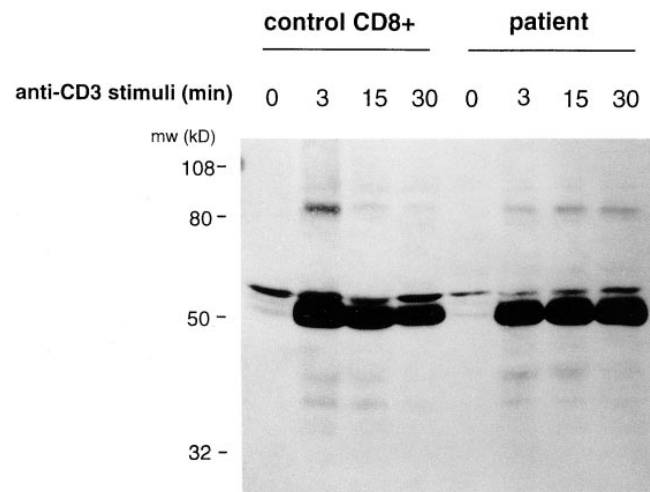
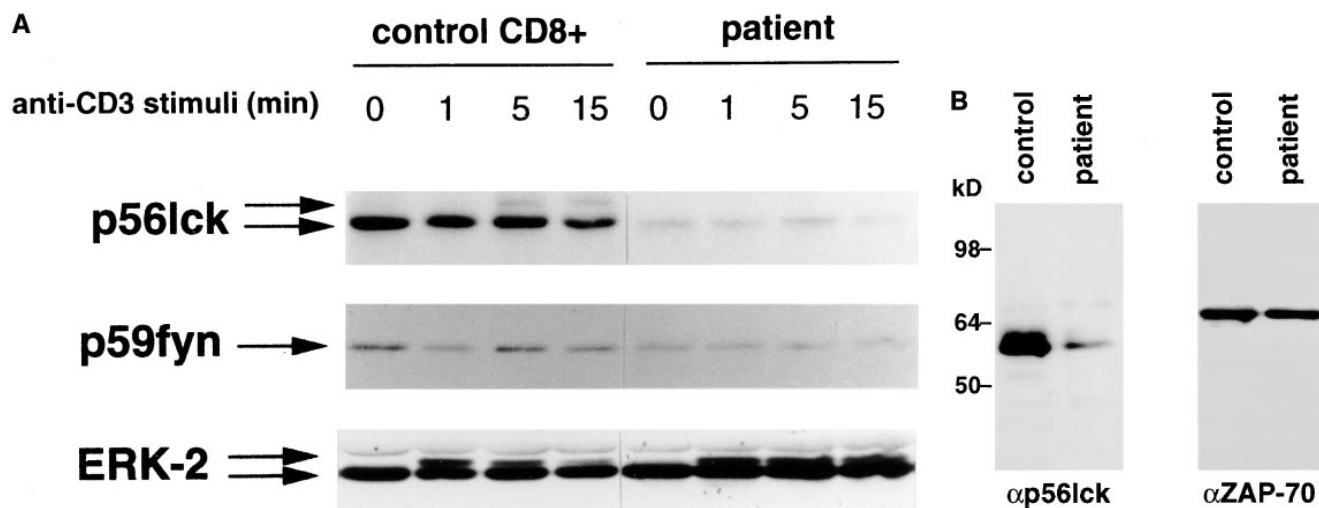


Figure 1. CD3-induced protein tyrosine phosphorylation in patient and control T cells. PBMC ( $10^6$ ) from the patient or a control donor (enriched for CD8<sup>+</sup> T cells by negative selection) were treated with anti-CD3 (OKT3, 40  $\mu$ g/ml) plus goat anti-mouse Ig (1:100) for various times, and whole well lysates were separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose, immunoblotted with an antiphosphotyrosine antibody (4G10), and immunoreactive bands were visualized by enhanced chemiluminescence.



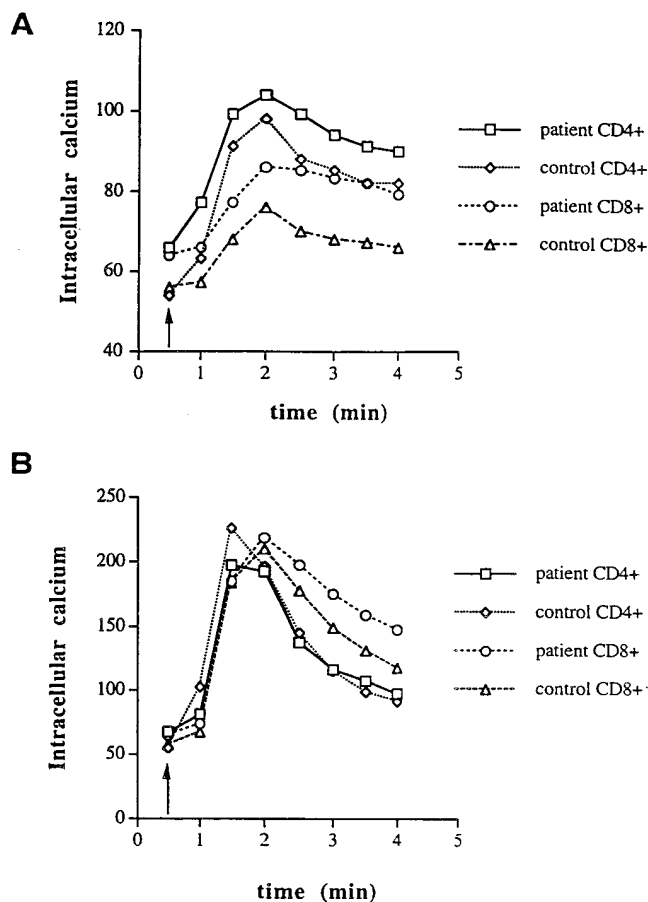
**Figure 2.** Expression of T cell-specific protein tyrosine kinases and ERK-2 activation. (A) PBMC ( $10^6$ ) from the patient or a control donor (enriched for CD8<sup>+</sup> T cells by negative selection) were treated with anti-CD3 (OKT3, 40  $\mu$ g/ml) plus goat anti-mouse Ig (1:100) for the indicated times, and proteins were separated by 10% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose, and membranes were blotted with either anti-p56lck (top), anti-p59fyn (middle), or anti-ERK-2 (bottom), and immunoreactive bands were visualized by BCIP/NBT. (B) T cell lysates from the patient and a control were probed with a polyclonal anti-lck antibody and after stripping, the same blot was reprobed with a polyclonal anti-ZAP-70 antibody to assess the relative levels of a T cell specific PTK. Bands were visualized by enhanced chemiluminescence.

*tient lymphocytes.* Next, we assessed expression and activation of individual PTKs known to be involved in the signal transduction cascade. The fyn PTK, a lymphocyte restricted member of the Src family, as well as the related ZAP-70 and Syk kinases, were all expressed at equivalent levels in patient and control cells (Fig. 2 and data not shown). In contrast, p56lck was markedly reduced in the patient T cells. To ensure that these results were not related to differences in the number of T cells present in the detergent lysates, nitrocellulose membranes were first immunoblotted with a polyclonal antibody against p56lck and then reprobed with a T cell specific ZAP-70 monoclonal antibody (Fig. 2 B). Consistent with the data in Fig. 2 A, expression of p56lck was significantly diminished in the patient cells, even though the relative levels of ZAP-70 were approximately equivalent in control and patient T cells. Indeed, densitometric analysis of several individual experiments revealed that the level of p56lck in the patient T cells was always < 10% of that observed in control T cells.

*Proximal TCR signaling events.* p56lck-deficient mice demonstrate a substantial reduction in the maturation of  $\alpha\beta$  T cells. Interestingly, those T cells that develop are able to proliferate, albeit at a reduced level, in response to CD3 and TCR $\alpha\beta$  cross-linking (13, 26, 27). Thus, proximal TCR signaling events were assayed in the patient T cells, including MAPK phosphorylation and calcium mobilization. T cell activation through the TCR is known to activate the ras signaling pathway, with resultant threonine and tyrosine phosphorylation of the MAP kinases ERK-1 and ERK-2. In both patient and control T cells, a band shift in ERK-2, a result of its phosphorylation, was observed after stimulation with OKT3 and cross-linking antibodies (Fig. 2 A). Additionally, mobilization of intracellular calcium, a proximal TCR signaling event required for protein kinase C (PKC) activation, proceeded normally in patient T cells. To monitor intracellular calcium flux within the differ-

ent T cell subsets, mononuclear cells from the patient and a control were stained with FITC-labeled anti-CD4 or anti-CD8 antibodies (Fig. 3 A). CD3 cross-linking (biotinylated OKT3 plus avidin) of CD4<sup>+</sup> or CD8<sup>+</sup> T cells isolated from both a control and the patient resulted in a modest increase in intracellular calcium, peaking at 90 s after stimulation. As previously described, TCR-mediated calcium changes were greater in CD4<sup>+</sup> than in CD8<sup>+</sup> cells (28). However, the CD3-induced calcium flux in the patient CD8<sup>+</sup> cells was similar to that observed in control CD8<sup>+</sup> cells. Stimulations were also carried out by simultaneously adding OKT3 plus secondary cross-linking goat anti-mouse antibodies, effectively co-cross-linking CD3/CD4 or CD3/CD8 (Fig. 3 B). This treatment resulted in a larger calcium flux in both control and patient T cell subsets. Thus, the lack of p56lck did not appear to diminish either CD3-induced phosphorylation of ERK-2 or calcium flux. These data indicate that several proximal TCR signaling events are intact in thymocytes from p56lck-deficient mice and in peripheral T cells from this patient with low p56lck expression (11, 13, 26, 27, 29).

*Low expression of p56lck prevents CD3-mediated CD69 expression.* CD69 is an early activation antigen that is normally upregulated in lymphocytes within 24 h of TCR ligation or exposure to phorbol esters (30, 31). Mononuclear cells from the patient and a control were cultured overnight in the presence of an immobilized anti-CD3 antibody (OKT3) or PMA plus ionomycin, then stained with FITC-labeled anti-CD69 and PE-conjugated anti-CD8 antibodies. As demonstrated in Fig. 4, stimulation with either immobilized OKT3 or PMA plus ionomycin upregulated CD69 expression in control CD8<sup>+</sup> T cells, as indicated by a one to two log shift in relative fluorescence compared with nonstimulated cells. Consistent with earlier reports, the highest level of CD69 expression was observed in cells treated with PMA plus ionomycin (30, 32). In contrast,



**Figure 3.** Decreased expression of p56lck does not affect CD3-induced calcium flux. PBMC from the patient and a normal control were loaded with the calcium binding Indo-1 dye, stained with FITC-conjugated mouse anti-human CD4 (Leu 3a) or mouse anti-human CD8 (Leu 2a), and stimulated (as indicated by arrows) with (A) biotinylated OKT3 (40  $\mu$ g/ml) plus avidin, or (B) OKT3 (40  $\mu$ g/ml) plus goat anti-mouse Ig, effectively co-cross-linking CD3/CD4 or CD3/CD8 receptors. Changes in the ratio of fluorescence at 390 and 515 nm, which correlate with intracellular calcium concentration (arbitrary units), were plotted over time (minutes).

although CD69 expression was increased on patient CD8<sup>+</sup> T cells after treatment with PMA plus ionomycin, no increase in CD69 expression was observed following OKT3 stimulation. The finding that CD69 expression in the patient CD8<sup>+</sup> T cells was induced after treatment with PMA plus ionomycin but was not detected after CD3 engagement suggests that the TCR signaling defect lies proximal to PKC activation.

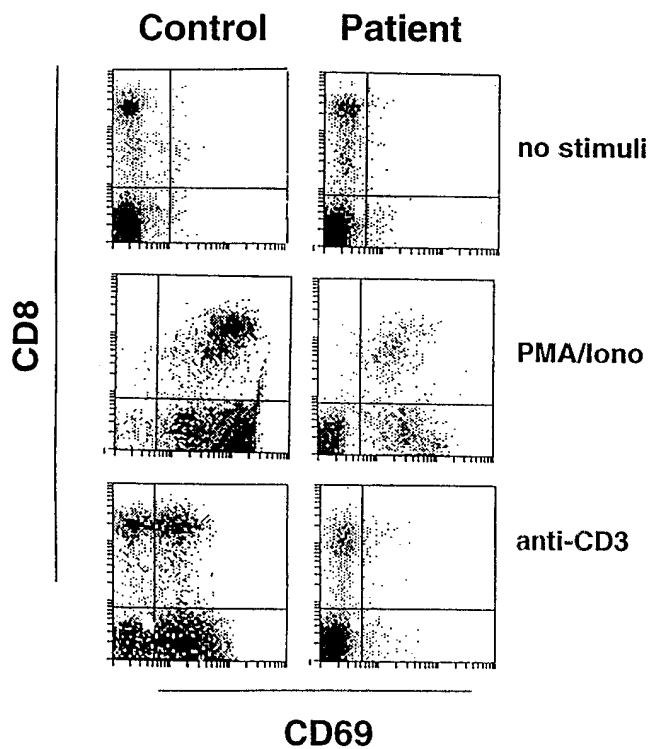
**Defective proliferative responses in lymphocytes expressing low levels of p56lck.** Proliferation of T cells after exposure to various mitogens, alloantigens, IL-2, and TCR engagement is crucial for the development of an intact immune response. As noted in Table III, the proliferative responses of the patient T cells to anti-CD3, IL-2, and mitogens were consistently decreased relative to control peripheral mononuclear cells. Since CD8<sup>+</sup> T cells comprised a majority of the lymphocyte population in the patient, proliferative responses were also compared with enriched control CD8<sup>+</sup> T cells. In this case as well, the level of blastogenesis was significantly higher in the control CD8<sup>+</sup> cells than in the patient cells after stimulation.

Moreover, during a 26-mo period, the patient demonstrated a progressive decline in overall T cell responsiveness to mitogens and anti-CD3 antibodies. While proliferative responses to anti-CD3, PHA, and Con A were 2.5- to 5-fold lower than controls at 4 mo of age, these responses were 6- to 20-fold lower than control levels by 20 mo of age. Interestingly, during this same time period, proliferative responses of the patient cells to alloantigen were intact. These data suggest that intact expression of p56lck may be required for an optimal response to mitogenic, but not alloantigenic, stimuli.

**Exon 7 is lacking in lck transcripts from the patient.** p56lck negative T cells (JCaM.1) contain lck transcripts lacking exon 7, suggesting that exon 7 is necessary for protein stability and expression (33). Thus, experiments were carried out to determine if the loss of p56lck in the patient was due to a defect at the transcriptional level. To more precisely examine the structure of the lck mRNA, cDNA was produced by RT-PCR using lck specific oligonucleotide exon primers from the 5' and 3' ends as well as from within the exon 7 kinase domain. As shown in Fig. 5 (left and right panels), RT-PCR products generated from the patient's cDNA using forward and reverse primers located within the 5' and 3' ends of the molecule yielded two bands of approximately equal intensity, whereas the lower band was barely detectable or absent when cDNA from control PBMC, the parents, or Jurkat cDNA was used.

**Table III. In Vitro Proliferative Responses—Stimulation Index (cpm)**

Stimuli	Age (mo)	Lab control	Patient	CD8 <sup>+</sup> control
Alone	4	1 (429)	1 (717)	
	6	1 (677)	1 (697)	
	20	1 (835)	1 (2560)	1 (437)
	30	1 (179)	1 (126)	
PWM	4	123 (52672)	14 (9876)	
	6	110 (74733)	10 (6971)	
	20	12 (9946)	2 (3850)	11 (4680)
Con A	4	350 (150263)	66 (47274)	
	6	162 (110113)	13 (9219)	
	20	85 (70701)	6 (16611)	109 (47489)
PHA	4	404 (173153)	151 (107973)	
	6	194 (131137)	40 (27564)	
	20	55 (45428)	8 (20826)	95 (41497)
IL-2	4	78 (33601)	21 (14962)	
	6	54 (36640)	13 (8950)	
	20	38 (32005)	11 (27994)	67 (29089)
Anti-CD3	4	198 (84771)	81 (58133)	
	6	113 (76953)	56 (39265)	
	20	34 (28311)	9 (22197)	68 (29824)
	30	240 (43137)	9 (1152)	
Alone	6	1 (714)	1 (104)	
	18	1 (297)	1 (210)	1 (649)
	30	1 (320)	1 (83)	
Alloantigen	6	76 (54337)	106 (11028)	
	18	84 (24933)	131 (27593)	49 (31822)
	30	148 (47340)	119 (9915)	



**Figure 4.** p56lck is required for CD3-mediated CD69 expression. PBMC from the patient and a normal control were cultured for 24 h in 24-well plates ( $2 \times 10^6$ /ml) in medium alone or in the presence of PMA (50  $\mu$ g/ml) plus ionomycin (0.5  $\mu$ M), or immobilized OKT3 (3  $\mu$ g/ml). Cells were then harvested and stained with FITC-conjugated anti-CD69 (Leu 23) and PE-conjugated anti-CD8 (Leu 2). Fluorescence intensity was measured on an EPICS 753 and results are representative of data obtained in three separate experiments.

Additionally, a similar pattern of predominance of this upper band and a near complete absence of a lower band was noted in 20 additional controls when cDNA was amplified with the most 5' and 3' *lck* primers (data not shown). When forward or reverse primers located within exon 7 were used (Fig. 5, middle panel), only a single band was seen in the PCR reaction using patient or control cDNA. This suggests that the predominant lower band observed in the patient lacked exon 7 and

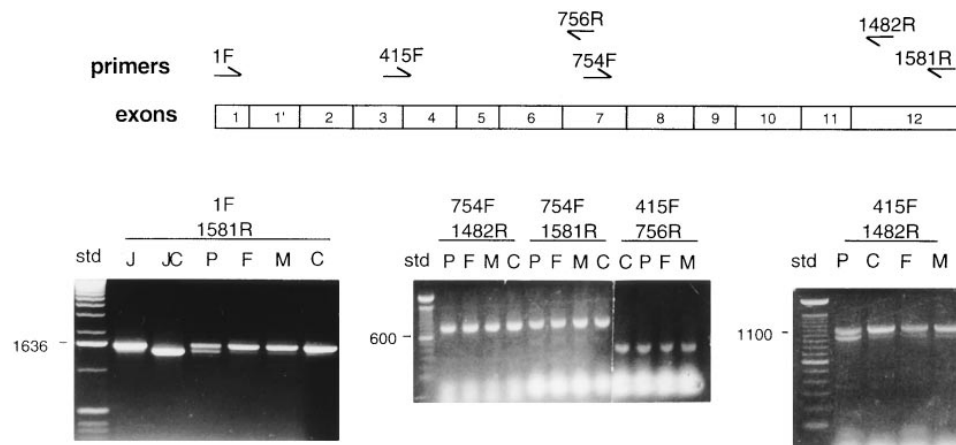
thus could not serve as a template for PCR reactions using primers within this region. A fragment with the molecular mass of the lower band was observed when cDNA from the p56lck-deficient JCaM.1 cell line was used as a template (Fig. 5, left panel, lane 2). The upper and lower bands from the patient were purified and PCR products were sequenced. Interestingly, all sequences normally found in exon 7 were absent from the lower band. To test whether the skipping of exon 7 was due to splice site mutations, intron 6, exon 7, and intron 7 were PCR amplified from patient and control genomic DNA and then sequenced. No differences were observed in the splice sequences flanking exon 7 nor within 20 bp on either side. Of note, no smaller *lck* protein was observed in the patient's cells or JCaM.1 cells using two p56lck-specific antibodies that recognize distinct epitopes upstream of exon 7. This suggests that an exon 7 deleted *lck* protein was unstable and/or that conformational changes precluded detection with these antibodies.

### Discussion

We describe here a patient with SCID and selective CD4 lymphopenia. The patient had only minimal p56lck protein expression and exhibited an unusual mRNA splicing pattern of the *lck* gene. While mice deficient in p56lck have been reported, this is the first demonstration of a human SCID patient with an abnormal expression of p56lck.

One of the unusual features of this patient is the predominant loss of CD4<sup>+</sup> T lymphocytes. Although there have been reports of adults with idiopathic CD4 lymphopenia who display many symptoms of acquired immunodeficiency, T cells from these patients are functional and immunoglobulin synthesis is normal (34). There has also been one described case of a selective congenital deficiency of CD4<sup>+</sup> T cells, and like the patient described here, that child exhibited profoundly decreased blastogenesis and IL-2 secretion in response to mitogenic stimuli (35). However, the etiology of CD4 lymphopenia in that child was never reported.

Another unique feature of the patient described here was the decreased expression of CD28 on CD8<sup>+</sup> T cells. While CD28 is normally expressed on 70–90% of CD8<sup>+</sup> T cells (36), it was only expressed on 2–7% of patient CD8<sup>+</sup> T cells. The CD28 molecule has been shown to be important for immune competence as CD28 knockout mice demonstrate impaired T



**Figure 5.** Aberrant splicing of *lck* transcripts in patient and JCaM.1 T cells. RNA isolated from PBMC from the patient (P), mother (M), father (F), and an unrelated normal healthy control (C), as well as from Jurkat (J), and JCaM.1 (JC) was reverse transcribed and cDNA was used in subsequent PCR reactions. PCR primers were derived from the published human sequence and are located within the 5', internal, and 3' regions of the *lck* cDNA, as shown in the upper diagram. *lck* cDNA was amplified using the indicated primer pairs and products were analyzed by agarose gel electrophoresis.

cell responses to lectins and immunoglobulin synthesis is diminished, though T cell development appears to be intact (37). Moreover, there is an increased frequency of the CD8<sup>+</sup>/CD28<sup>-</sup> T cell subset in HIV-infected individuals (38, 39) and CD28 expression is also downmodulated in CD4<sup>+</sup> T cells from patients with rheumatoid arthritis (40). These latter cells lack CD7 expression and exhibit an unusual functional profile in that they are autoreactive to ubiquitously distributed autoantigens. Of note, our patient also had low to nearly absent expression of CD7 (data not shown) on CD8<sup>+</sup> T cells. Although p56lck is believed to phosphorylate CD28 and is required for binding of the ITK protein kinase to CD28, the relationship between diminished p56lck levels and CD28/CD7 expression on the patient's CD8<sup>+</sup> T cells remains to be determined.

While the precise genetic defect responsible for the decreased expression of p56lck in our patient is still not fully understood, it is likely due to aberrant alternative splicing of exon 7 of the *lck* gene. As previously observed, the Jurkat-derived JCaM.1 line, which does not express any detectable p56lck and exhibits defective TCR signaling, is also lacking exon 7 within the *lck* transcript. The genetic mutation responsible for deletion of exon 7 in JCaM.1 cells has never been determined, although it is noteworthy that the parental Jurkat cells also express very low levels of this shortened *lck* transcript. Our finding that this mutant transcript accounts for a significant proportion of the specific *lck* mRNA in the patient described here suggests that alternative splicing of exon 7 may be critical in regulating p56lck protein expression. Protein instability may account for the decreased expression of the mutant (exon 7 deleted) *lck* protein. Likewise, mutations within the kinase domain of ZAP-70 result in diminished or absent levels of ZAP-70 protein expression (7, 8, 41). However, in our patient, a wild-type *lck* mRNA transcript was also observed. Thus, the disproportionate decrease in *lck* protein might be explained by hypothesizing that an exon 7-deleted *lck* transcript behaves in a dominant negative fashion, resulting in the downmodulation of wild-type p56lck. Indeed, levels of *lck* protein in this patient were < 10% of those observed in control T cells.

Since p56lck associates directly with CD4 or CD8 at the surface of the T cell and is one of the first kinases to be activated upon TCR engagement (42–44), it is noteworthy that proximal TCR signaling could be observed in the patient T cells. Tyrosine phosphorylation of cellular substrates, one of the earliest events in the TCR signaling cascade, was intact in patient T cells after CD3 cross-linking. Interestingly though, high levels of protein tyrosine phosphorylation were also observed upon CD3 stimulation of CD4<sup>+</sup>/CD8<sup>+</sup> thymocytes isolated from p56lck-deficient mice (17). Collectively, these data suggest that proximal signaling responses in T cells may be elicited in the presence of low levels of p56lck.

Further dissection of the signaling pathway in T cells from this patient indicated that although anti-CD3 induced proliferation and CD69 expression were severely affected, calcium mobilization was intact after TCR engagement. These results suggest that early TCR responses may be uncoupled from a full cellular response. This is further supported by the observation that in CD4<sup>+</sup> T cells isolated from SCID patients with ZAP-70 deficiency, early TCR-mediated events such as calcium flux and activation of PLC- $\gamma$ 1 are only partially affected while later events such as IL-2 production and TCR-mediated proliferation are completely abrogated (9).

Mice with complete deletions of p56lck have a slightly dif-

ferent phenotype from the patient described in this report, with a substantial reduction in the number of CD4<sup>+</sup>/CD8<sup>+</sup> thymocytes (13, 16). This may be due to a complete absence of p56lck in the knock-out mice whereas there is low expression of this kinase in our patient. Although the development of only a few mature  $\alpha\beta$  cells has been observed in *lck* null mice, it is interesting that the ratio of single positive CD8/CD4 cells in the thymus, lymph node, and spleen in these animals ranges from 2–3:1 (17, 18). This is in sharp contrast to the situation observed in wild-type mice where the ratio of single positive CD8/CD4 cells in the thymus is 1:4 and the CD8/CD4 ratio in the lymph node and spleen is approximately 1:2 (17, 18). These data indicate that while the development of mature T cells is more severely affected in the *lck* null mice than in our patient, in both cases development of CD8<sup>+</sup> cells is favored. Thus, a deficiency in p56lck appears to have a greater detrimental impact on the maturation of CD4<sup>+</sup>  $\alpha\beta$  T cells than on CD8<sup>+</sup>  $\alpha\beta$  T cells.

Recent work has shown that the p59fyn protein kinase, which is expressed in both immature and mature T lymphocytes, partially compensates for the lack of p56lck in T cell development. T cell development is completely arrested at the CD4<sup>+</sup>/CD8<sup>-</sup> thymocyte stage in mutant mice that lack p56lck and p59fyn (17, 18). The patient in this study expressed normal levels of p59fyn and it is therefore possible that p59fyn played a role, albeit incomplete, in the development of his mature T cells. The observation that p56lck and p59fyn have distinct intracellular distributions in T cells has been proposed as one of the reasons why they are not completely redundant in T cell signaling and development (45). However, it remains to be determined why T cell development in the presence of p59fyn, with absent or low levels of p56lck, appears to favor the maturation of CD8<sup>+</sup> T cells in both mice and humans. In this regard, it is intriguing to note that there is some data indicating that the regulation and activation of p56lck differ in CD4<sup>+</sup> and CD8<sup>+</sup> cells. In vitro, p56lck undergoes less vigorous phosphorylation when bound to CD8 as compared with CD4 and upon PKC activation, p56lck dissociates from CD4 but remains bound to CD8 (46).

Finally, recent data have demonstrated that although signaling is not observed in a Jurkat T cell line that is deficient in functional p56lck but expresses ZAP-70, TCR signaling can be reconstituted after introduction of the tyrosine kinase, Syk (47). In light of these results, it is interesting to note that Syk expression was detected in the patient's T cells and moreover was tyrosine phosphorylated upon CD3 cross-linking (data not shown). Therefore, the ability of kinases such as Syk to be activated in an *lck*-independent manner may also result in a partial compensation for the diminished level of the latter kinase in the patient's T cells. However, the selective CD4 lymphopenia in our patient and the impaired ability of his T cells to proliferate in response to mitogens or TCR stimulation indicates activation of Syk was not sufficient to mediate a full cellular response in these primary T cells. Collectively our results demonstrate that intact expression of p56lck is important in promoting both normal thymopoiesis and T cell responsiveness in humans.

Comparisons of the defects in humans and mice lacking ZAP-70 indicate that the effects are not identical. T cell development is blocked at the CD4<sup>+</sup>/CD8<sup>+</sup> thymocyte stage in mice deficient in ZAP-70 while humans with ZAP-70 deficiency develop non-functional CD4<sup>+</sup> T cells (7–9, 41, 48). We now dem-

onstrate that a patient expressing very low levels of p56lck has a selective CD4 lymphopenia while mice deficient in p56lck show a substantial reduction in CD4<sup>+</sup>/CD8<sup>+</sup> thymocytes with the development of only a small number of peripheral single positive T cells. Further studies of humans and mice with mutations in the genes encoding these PTKs will enable us to better understand their roles in human and murine T cell development.

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