TGase II cDNA (pMT2′rl TGase) (Fig. 2) or with a plasmid (pCMV5) containing a $G\alpha_q$ cDNA (*10*) (6 to 8 μg per 5 × 10^6 cells) by the DEAE-dextran method; after 48 to 72 hours, cells were harvested and membranes prepared as described [D. M. Perez, M. B. DeYoung, R. M. Graham, *Mol. Pharmacol.* **44**, 784 (1993); R. R. Franke, T. P. Sakmar, R. M. Graham, H. G. Khorana, *J. Biol. Chem.* **267**, 14767 (1992)]. Membranes or purified $G\alpha_h$ were subjected to SDS–polyacrylamide gel electrophoresis (8% polyacrylamide). The proteins were transferred to nitrocellulose filters, which were then incubated in 5% (w/v) dried milk and probed with the various antibodies diluted 1:500. Bound antibodies were detected with ^{125}I-labeled protein A (ICN) (polyclonal antisera), or with rabbit antibodies to mouse immunoglobulin G followed by ^{125}I-labeled protein A (monoclonal antibodies). The blots were subjected to autoradiography with Kodak XAR-5 film and image intensifying screens (Dupont).

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9. Single-stranded cDNA was prepared from 20 μg of rat liver total RNA with Moloney murine leukemia virus reverse transcriptase, and PCR was then performed on one-half of the sample [D. M. Perez, M. T. Piascik, R. M. Graham, *Mol. Pharmacol.* **40**, 876 (1991)] with primers based on the mouse, human, and guinea pig TGase II 5′ (translation start site) and 3′ (stop site) coding regions (*16*). The resulting PCR product (~2.1 kb) was cloned into the modified eukaryotic expression vector pMT2′ to yield pMT2′rlTGase. After amplification in *Escherichia coli* (DH5α cells), CsCl-purified plasmid DNA was prepared and used both to determine the nucleotide sequence of the entire PCR product and for transfection studies (Fig. 3).
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18. TGase activity was determined by quantitating the incorporation of [^{3}H]putrescine into *N,N′*-dimethylcasein as described by Achyuthan and Greenberg (*5*). Membranes (25 to 30 μg of protein) in 50 μl of tris-HCl (pH 7.4) containing 1 mM $MgCl_2$, 20 mM dithiothreitol, and 20% (v/v) glycerol were incubated with 0.4% *N,N′*-dimethylcasein, 2 μCi of [^{3}H]putrescine, and various indicated additions for 1 hour at 37°C. The reactions were stopped and the radioactivity incorporated was determined as described (*5*).
19. Buffalo rat liver cells (American Type Culture Collection) or COS-1 cells were transfected with 6 to 8 μg of each plasmid per 5 × 10^6 cells. After 48 to 72 hours, the effects of (−)epinephrine and prazosin on [^{3}H]inositol phosphate accumulation were assayed in buffalo rat liver cells (*1*), and membranes were prepared from COS-1 cells. Membranes were assayed for [^{3}H]PIP_2 hydrolysis after treatment with (−)epinephrine (1 μM), or epinephrine plus prazosin (1 μM), essentially as described (*15*), except that the concentrations of GTP-γ-S and GDP-β-S were 1 and 50 μM, respectively.
20. An anti–TGase II antibody affinity column (50 μl) was prepared by incubation of protein A–agarose with antibodies to TGase II for 2 hours at 4°C. The gel was washed extensively with 20 mM Hepes (pH 7.4) containing 100 mM NaCl, 0.5 mM dithiothreitol, 5% (v/v) glycerol, and 0.05% sucrose monolaurate. A ternary complex of α_1-agonist, receptor, and $G\alpha_h$ (300 fmol of α_1-receptor, 330 fmol of $G\alpha_h$), prepared as previously described (*1*), was incubated with the resin at 4°C for 2 hours in the presence of 1 mM $MgCl_2$, and either 5 μM (−)epinephrine, 5 μM (−)epinephrine and 5 μM GTP-γ-S, or 5 μM (−)epinephrine and 0.1 mM phentolamine. Unbound material was eluted from the column by applying gentle pressure until the column was dry. After the resin was washed with the same buffer (3 to 5 ml) in the absence of ligand, adsorbed α_1-adrenergic receptor was eluted by incubating the gel with 2 μM (−)epinephrine, 5 μM GTP-γ-S, and 1 mM $MgCl_2$ for 30 to 60 min at 4°C. Receptor density in the flow-through and eluted fractions was determined after removing excess ligand on a dried Sephadex G-25 (Pharmacia) column (3 ml) as described (*1, 2*).
21. The 69-kD PLC (100 ng) and either guinea pig liver TGase II (500 ng) or G_{h7} (1 μg) were purified and reconstituted into phospholipid vesicles as described (*2, 3, 7*). The vesicles were incubated with 1 mM $MgCl_2$ in the absence or presence of 5 μM GTP-γ-S at 30°C for 30 min before evaluation of PLC activity. The samples were then incubated at 30°C for 10 min in the presence of [^{3}H]PIP_2 (500 cpm/nmol) and various concentrations of Ca^{2+}, and IP_3 formation was measured as described (*2*).
22. We thank P. J. Birckbichler for the antibodies to guinea pig liver TGase II; P. C. Sternweis for antibodies to $G\alpha_q$; G. L. Johnson for the $G\alpha_q$ cDNA clone; J. R. Shainoff for a sample of purified guinea pig liver TGase II; G. Matsueda for valuable discussions; R. Gaivin, T. Tang, and R. P. Riek for technical assistance; and S. Cox for typing the manuscript. Supported by NIH (A.H., M.-J.I., and R.M.G.), a grant-in-aid (D.M.P.) and postdoctoral fellowship (H.N.) from the American Heart Association (Northeast Ohio Affiliate), and an Eccles Award (R.M.G.) from the National Health and Medical Research Council of Australia.

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Human Severe Combined Immunodeficiency Due to a Defect in ZAP-70, a T Cell Tyrosine Kinase

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A homozygous mutation in the kinase domain of ZAP-70, a T cell receptor–associated protein tyrosine kinase, produced a distinctive form of human severe combined immunodeficiency. Manifestations of this disorder included profound immunodeficiency, absence of peripheral $CD8^+$ T cells, and abundant peripheral $CD4^+$ T cells that were refractory to T cell receptor–mediated activation. These findings demonstrate that ZAP-70 is essential for human T cell function and suggest that $CD4^+$ and $CD8^+$ T cells depend on different intracellular signaling pathways to support their development or survival.

Severe combined immunodeficiencies (SCIDs) are a heterogeneous group of inherited disorders characterized by profound deficiency of both T cell and B cell immunity (*1*). In about 30% of cases, the underlying genetic defect is unknown. We recently identified a 1-year-old girl (the daughter of first cousins) in whom SCID was associated with a highly unusual T cell subset distribution in the blood (*2*). T cells are normally divided into two major subsets that express either CD4 or CD8 protein on their surfaces. In our patient, the overall T cell count was moderately elevated, but $CD8^+$ cells were virtually absent and nearly all circulating T cells were of the $CD4^+$ type (Fig. 1).

The patient's $CD4^+$ T cells were polyclonal, expressed other surface markers of mature lymphocytes, and proliferated normally when exposed to a combination of phorbol ester and calcium ionophore in vitro. However, they did not respond to mitogenic stimuli mediated through the surface T cell receptor (TCR)–CD3 complex, such as CD3-CD4 co-crosslinking, or to allogeneic stimulation in a culture of mixed lymphocytes. An earlier report (*3*) described similar findings in children from a Mennonite kindred and attributed the abnormalities to an undetermined defect in TCR signal transduction. As was consistent with this view, CD3-CD4 co-crosslinking of our patient's cells failed to trigger the cascade of cytoplasmic protein tyrosine kinase (PTK) reactions that is an early, obligatory step in T cell activation (*2, 4*). This implied a functional defect in coupling of the TCR to cytoplasmic PTKs.

We therefore used protein immunoblots to assay for individual PTKs that have been implicated in TCR signal transduction, including Lck, Fyn, and the T cell–specific kinase ZAP-70. ZAP-70 is a 70-kD cytosolic kinase that associates with the ζ chain of the TCR complex soon after this complex binds a ligand, and it has been proposed that it participates in T cell activation (*5–7*). Normal amounts of Lck and Fyn were present in our patient's cells, but no ZAP-70 protein was detectable (Fig. 2A).

A Northern (RNA) blot of the patient's T cell RNA showed ZAP-70 mRNA of

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approximately normal size and abundance. We cloned ZAP-70 complementary DNA (cDNA) from this RNA and then determined the complete coding sequences from two independent clones and partial sequences from two others. All four cDNA clones had sequences identical to wild-type sequences except that each contained a 13–base pair (bp) deletion involving nucleotides 1719 to 1731 (Fig. 2B). This deletion would be expected to cause a translational frameshift after amino acid 503, resulting in premature termination 35 codons downstream and yielding a mutant protein 82 residues shorter than wild-type ZAP-70 (Fig. 2C). Moreover, the frameshift occurs within the ZAP-70 kinase domain (*7*) and would eliminate at least two residues (Trp^{505} and Glu^{509}) that are strictly conserved among known kinase domains and are essential for PTK activity (*7*, *8*).

We tested the effect of this mutation by transfecting cultured avian cells with expression vectors encoding either wild-type or mutant ZAP-70. Protein immunoblots of the transfected cells were prepared by use of an antiserum against a portion of ZAP-70 that is unaffected by the mutation. Whereas the wild-type vector yielded protein of the expected size, little or no ZAP-70 was observed in cells receiving the mutant vector (Fig. 2D). This suggests that the mutant protein is relatively unstable in vivo, which is consistent with the finding that our patient's T cells lacked ZAP-70 protein despite normal expression of ZAP-70 mRNA.

To verify that the deletion was encoded in the patient's genome, we amplified by polymerase chain reaction (PCR) the informative region in chromosomal DNA from the patient and from a normal control. The control yielded a 520-bp PCR product composed of a central 141-bp exon flanked by two short introns and by portions of two other exons (Fig. 3A). The PCR product from the patient's DNA was identical except that it contained the 13-bp deletion, which is located entirely within the central exon and removes a unique Rsa I restriction site. We found that Rsa I cleaved the control PCR product into two co-migrating halves but did not cleave PCR products from the patient's DNA (Fig. 3B), which indicates that she was homozygous for the ZAP-70 mutation.

We then examined the genes encoding ZAP-70 in all living first-degree relatives of our patient. The informative region was PCR-amplified from leukocyte DNA of both parents, three younger siblings, and an unrelated control, all of whom were immunologically normal. When PCR products were examined on an agarose gel (Fig. 4A), the patient's DNA (lane 4) yielded a single band that was slightly smaller than that of the control (lane 1), which confirmed that she was homozygous for the deletion. Each parent's DNA (lanes 2 and 3) yielded two bands that co-migrated with the control and mutant bands, respectively, which showed that both parents were heterozygous for the same mutation. Two unaffected siblings (lanes 5 and 7) also proved heterozygous, whereas the third (lane 6) appeared to carry two wild-type alleles. Analysis of these same PCR products on a nondenaturing polyacrylamide gel (Fig. 4B) confirmed this interpretation by showing the two expected heteroduplex PCR bands in all four heterozygous family members (9) and thus confirming that only the affected child lacked a normal allele encoding ZAP-70.

Our results define an inheritable syndrome

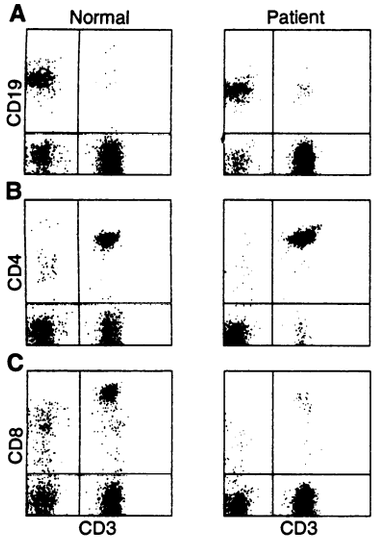

Fig. 1. Lymphocyte populations in the patient's peripheral blood (*2*) as compared with an age-matched control. The patient's total blood lymphocyte counts ranged from 10 to 20 × 10^3 cells per cubic millimeter, of which roughly 20% were CD3-$CD19^+$ B cells (**A**) and 75% were $CD3^+CD4^+$ T cells (**B**). The few $CD3^+CD8^+$ cells present (0 to 2% of total blood lymphocytes; normally 20 to 30%) were also $CD56^+$ (*17*) and were therefore judged to be NK cells (**C**).

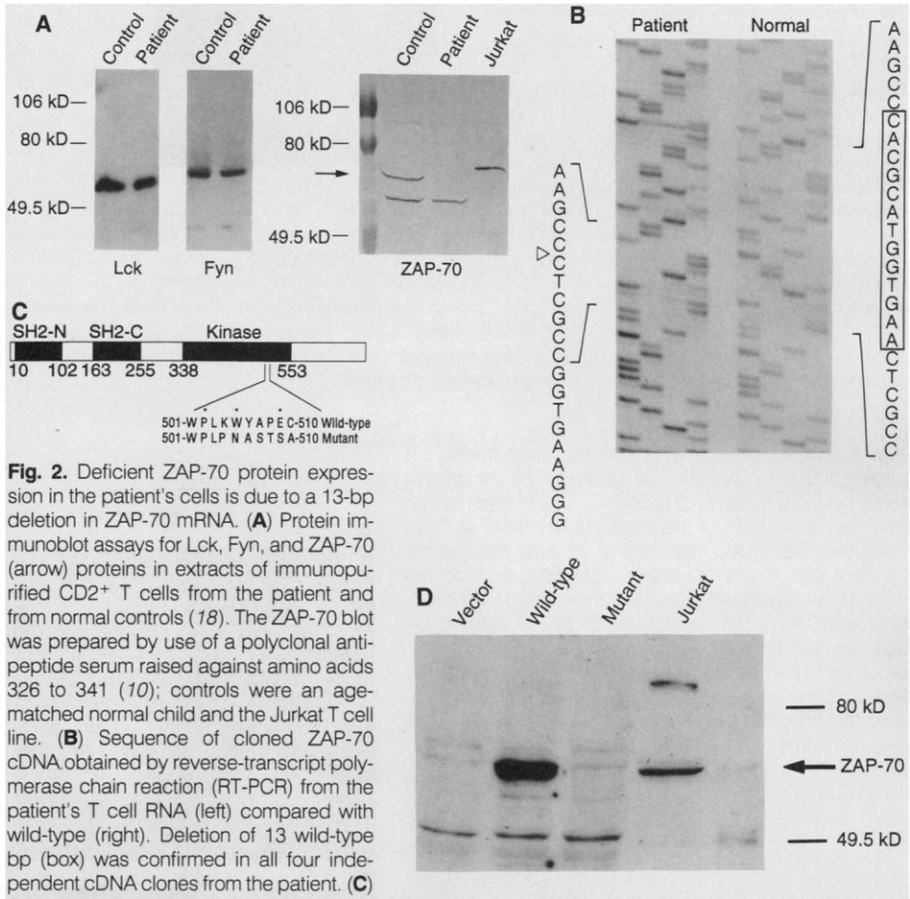

Fig. 2. Deficient ZAP-70 protein expression in the patient's cells is due to a 13-bp deletion in ZAP-70 mRNA. (**A**) Protein immunoblot assays for Lck, Fyn, and ZAP-70 (arrow) proteins in extracts of immunopurified $CD2^+$ T cells from the patient and from normal controls (*18*). The ZAP-70 blot was prepared by use of a polyclonal anti-peptide serum raised against amino acids 326 to 341 (*10*); controls were an age-matched normal child and the Jurkat T cell line. (**B**) Sequence of cloned ZAP-70 cDNA obtained by reverse-transcript polymerase chain reaction (RT-PCR) from the patient's T cell RNA (left) compared with wild-type (right). Deletion of 13 wild-type bp (box) was confirmed in all four independent cDNA clones from the patient. (**C**) Schematic depiction of wild-type ZAP-70 protein (*7*), showing two Src homology regions (SH2-N and SH2-C) and the kinase domain (*19*). Also shown are predicted sequences for the wild-type and mutant proteins at amino acids 501 to 510 in the kinase domain. Asterisks mark three residues in the wild-type sequence that are invariant among known kinase domains (*8*); the mutation eliminates two of these. (**D**) Protein immunoblot of ZAP-70 expression in untransfected Jurkat T cells and in QT6 quail cells transfected 48 hours previously with plasmids encoding wild-type or mutant ZAP-70. "Vector" is the same plasmid without coding sequences.

in which SCID and an abnormal peripheral T cell subset distribution are associated with a deficiency of the ZAP-70 kinase. In our patient, this deficiency is due to a frameshift mutation that eliminates essential residues from the ZAP-70 kinase domain (*5–8, 10*) and reduces the stability of the protein. The resulting abnormalities provide the first genetic evidence that ZAP-70 is required for TCR signal transduction and for human T cell function. Although earlier studies have demonstrated the need for Lck, and perhaps Fyn, in T cell signaling (*4, 11, 12*), no ZAP-70–deficient cell lines or animals have yet been described. Interestingly, mice that lack the TCR ζ chain (*13*) have reduced T cell counts but do not show the T cell subset abnormality seen in our patient. This suggests that ZAP-70 can carry out its essential role in T cell activation without binding ζ, perhaps because ZAP-70 also associates with other TCR components (*6*).

The most distinctive feature of this disorder is its disparate effect on the $CD4^+$ and $CD8^+$ subsets. The proliferation, survival, and maturation of thymic T cell precursors are all thought to depend absolutely on TCR-mediated signals (*14*), and mice lacking Lck are unable to produce T cells of either subset (*15*). A similar block in thymocyte development may account for the absence of circulating $CD8^+$ cells in our ZAP-70–deficient patient. However, this same patient's blood contained large numbers of apparently mature $CD4^+$ T cells that were completely unresponsive to TCR-mediated stimuli and yet presumably developed normally in the thymus. A likely interpretation, supported by recent studies in mice (*12, 16*), is that the human TCR uses distinct intracellular signaling pathways at different stages of lymphoid development. Specifically, our patient's condition suggests the hypothesis that ZAP-70 is required for signaling in mature $CD4^+$ T cells and $CD8^+$ thymocytes but is dispensable for signaling and thymic selection in $CD4^+$ thymocytes. Development and survival of the major human T cell subsets might thus depend on divergent pathways of PTK-mediated signal transduction emanating from the TCR complex.

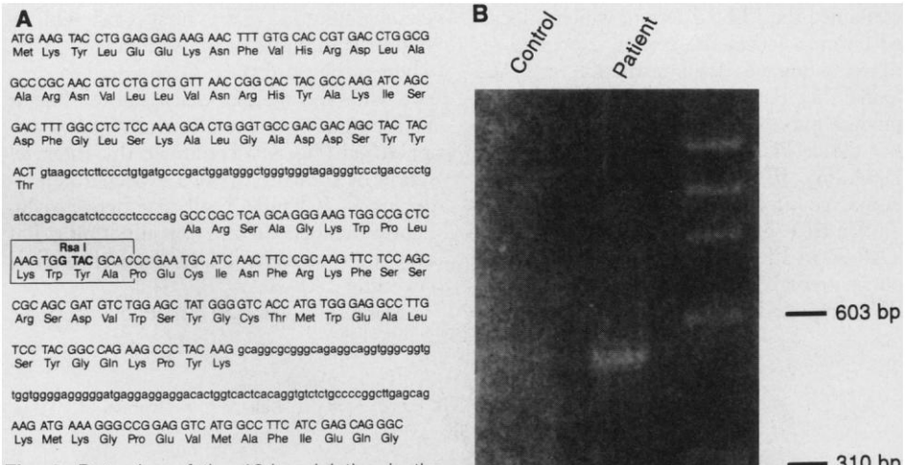

Fig. 3. Detection of the 13-bp deletion in the patient's genes encoding ZAP-70. (**A**) A portion of the wild-type gene encoding ZAP-70, showing the region deleted in the patient (box), which includes an Rsa I restriction site. Exon sequences are in uppercase letters, with predicted amino acid sequence below; introns are in lowercase letters. Sequences were determined by sequencing cloned PCR fragments prepared from leukocyte DNA from the patient and from a normal control, by use of primers 5′-CCTGATCCAGCAGCATCTCCC-3′ and 5′-GCCTTCATCGAGCAGGGCAAG-3′, which amplify the region shown. (**B**) Absence of the Rsa I restriction site in the patient's chromosomal genes encoding ZAP-70. The PCR products, prepared directly from control and patient DNA with the primers described in (A), were digested with Rsa I and analyzed on a 2% agarose gel. Products from both samples were of approximately equal length before digestion.

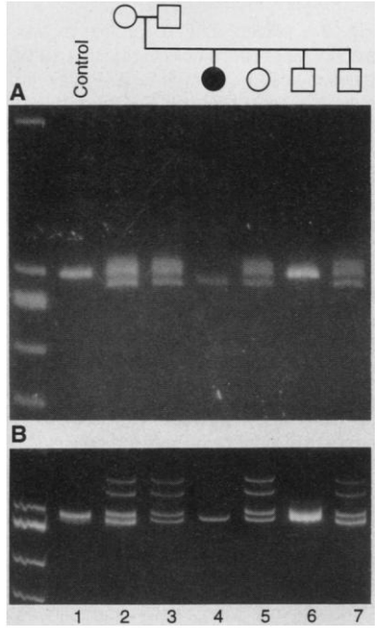

Fig. 4. Inheritance of wild-type and mutant alleles encoding ZAP-70 in the affected kindred. The informative region of the gene encoding ZAP-70 was PCR-amplified from leukocyte DNA of the index patient (lane 4), from three younger siblings (lanes 5 to 7), from the parents (lanes 2 and 3), and from an unrelated normal control (lane 1), as described (Fig. 3A). Undigested PCR products were analyzed electrophoretically on (**A**) a 4% agarose gel and (**B**) a non-denaturing 6% polyacrylamide gel. One additional male child in this kindred (the first born; not depicted) died in infancy after multiple recurrent infections, presumably as a result of SCID; no DNA from that child was available for analysis.

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2. Analysis of TCR β gene rearrangement and human lymphocyte antigen (HLA) typing confirmed that the patient's peripheral $CD4^+$ cells were polyclonal and of nonmaternal origin. These cells expressed normal levels of CD2, CD3, CD45, HLA classes I and II, interleukin-2 (IL-2) receptor, and TCR αβ proteins. They proliferated normally and secreted IL-2 when treated with phorbol myristate acetate plus ionomycin but did not respond to phytohemagglutinin, pokeweed mitogen, tetanus toxoid, anti-CD3, or allogeneic leukocytes. Functions and phenotypes of B cells, antigen-presenting cells, and natural killer (NK) cells appeared normal in vitro. The patient has since been cured of her disease by bone marrow transplantation.
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16. I. T. Chan *et al.*, *Science* **261**, 1581 (1993).
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18. Blood T cells used in all studies were purified by Ficoll-Hypaque centrifugation and CD2 immunoadhesion to magnetic beads. Total $CD2^+$-cell RNA was used for preparing oligo(dT)-primed reverse transcripts, which were then amplified by PCR. The PCR primers for full-length cDNAs (bases 164 to 2178) were 5′-GGACATCCACCTGTACGTCC-3′ (primer A) and 5′-GTTGTCTCCACACACAGCTG-3′ (primer B), derived from the 5′ and 3′ untranslated regions, respectively (*7*). Partial cDNAs were obtained by use of either primer B with 5′-ATGAAGTACCTGGAGGAG- AAG-3′ or primer A with 5′-GCCTTCATCGAGCAGGGCAAG-3′. The ZAP-70 expression vector pSV70 contained the full-length cDNA and an SV40 promoter (*7*); the mutant vector was prepared from pSV70 in a multi-step procedure whose effect was to substitute a 184-bp Hpa I–Stu I fragment of the wild-type cDNA with a fragment containing the mutation.
19. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; E, Glu; K, Lys; L, Leu; N, Asn; P, Pro; S, Ser; T, Thr; W, Trp; and Y, Tyr.
20. We are grateful to the patient and her family; to D. Umetsu, D. Wara, and M. Cowan for help in clinical evaluation; to D. Littman and A. Veillette, respectively, for the Lck and Fyn antisera; and to C. Crooks and D. Littman for valuable insights, advice, and comments on the manuscript. Supported in part by NIH grants AI29313 and GM43574 to T.G.P. and by RR01271, and HD-28825. M.E.E. is a Merck/American Federation for Clinical Research M.D.–Ph.D. Fellow.

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ZAP-70 Deficiency in an Autosomal Recessive Form of Severe Combined Immunodeficiency

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Protein tyrosine kinases (PTKs) play an integral role in T cell activation and differentiation. Defects in the Src-family PTKs in mice and in T cell lines have resulted in variable defects in thymic development and in T cell antigen receptor (TCR) signal transduction. Here, three siblings are described with an autosomal recessive form of severe combined immunodeficiency disease (SCID) in which ZAP-70, a non-Src PTK, is absent as a result of mutations in the ZAP-70 gene. This absence is associated with defects in TCR signal transduction, suggesting an important functional role for ZAP-70.

Four cytoplasmic PTKs have been implicated in TCR signaling: Lck, Fyn, ZAP-70, and Syk (*1*). Biochemical and genetic studies provide evidence for a role of the Src-family PTKs (*2*, *3*). A second family of PTKs, consisting of ZAP-70 and Syk, also has been implicated in TCR signaling (*4*–*9*). Members of these two PTK families interact with the TCR sequentially (*10*). Both ZAP-70 and Syk are recruited to the phosphorylated CD3 and ζ subunits after TCR stimulation (*3*–*10*). Phosphorylation of CD3 and ζ requires Lck (or possibly Fyn in some cells), suggesting that an Src-family PTK plays a role upstream of ZAP-70 or Syk in TCR signaling (*3*, *9*, *10*). Both the Src- and ZAP-70–Syk-families are required for the efficient induction of tyrosine phosphoproteins in heterologous cells (*6*, *10*), but the role of ZAP-70 or Syk is not clear. The function of ZAP-70 may be clarified by studying cells deficient in its expression.

Recently, a defect in the signaling events associated with TCR engagement was observed in patients with an autosomal recessive form of SCID (*11*, *12*). In an accompanying paper, one such patient was found to have a defect in ZAP-70 protein expression (*13*). This prompted us to study an unrelated family with children that have a similar clinical syndrome (*12*) for TCR signaling defects and to explore the possibility that ZAP-70 mutations could account for this SCID syndrome. Both parents and one child had normal lymphocyte numbers and function, but three of the four children (two boys and one girl) had a marked reduction in peripheral $CD8^+$ T cells with normal to elevated numbers of $CD4^+$ T cells (*12*). The $CD4^+$ cells failed to proliferate in response to phytohemagglutinin or concanavalin A (lectins that depend on TCR expression for mitogenic activity) or to CD3 monoclonal antibodies (mAbs). However, normal proliferative responses were observed to the combination of phorbol esters and calcium ionophores that

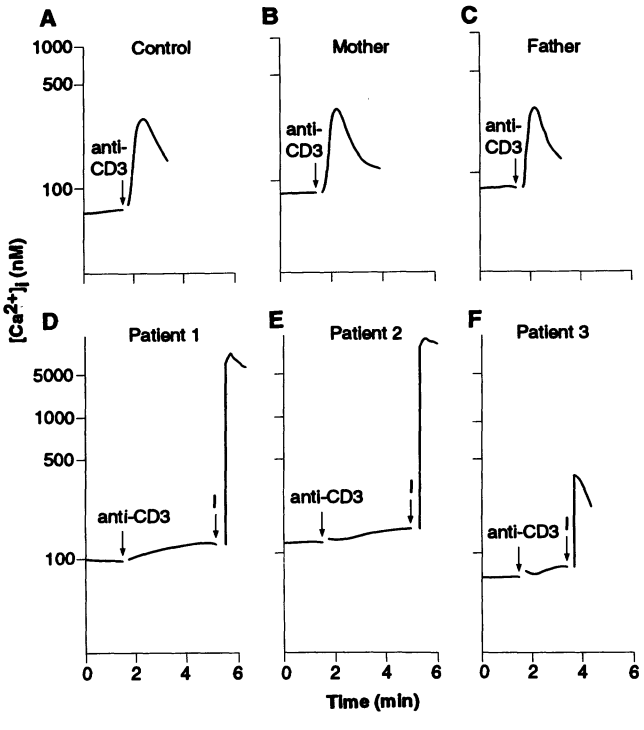

Fig. 1. Lack of mobilization of $[Ca^{2+}]_i$ in peripheral blood lymphocyte (PBL) lines from patients with SCID. PBL lines derived from (**A**) a normal donor (CD8-depleted), (**B**) the mother, (**C**) father, or (**D** to **F**) the three affected siblings were loaded with Indo-1 and cells cross-linked with the CD3 mAb, 235 (anti-CD3) (*18*). Ionomycin (I) was also added to nonresponding cells to ensure that cells were properly loaded with Indo-1.

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