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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

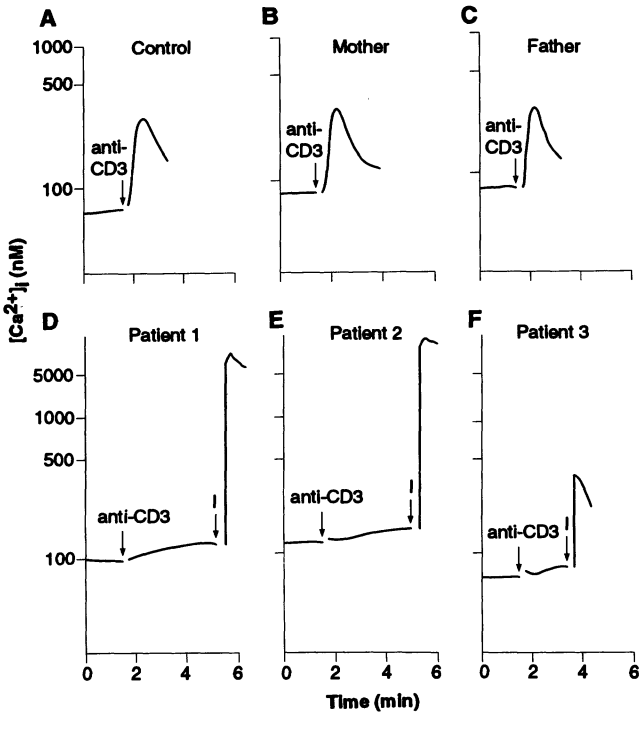

Fig. 1. Lack of mobilization of $[Ca^{2+}]_i$ in peripheral blood lymphocyte (PBL) lines from patients with SCID. PBL lines derived from (**A**) a normal donor (CD8-depleted), (**B**) the mother, (**C**) father, or (**D** to **F**) the three affected siblings were loaded with Indo-1 and cells cross-linked 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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mimic some of the initial events induced by the TCR (*12*). These patients had normal natural killer cell activity and levels of serum immunoglobulin. Expression of TCR, CD3, and CD45 was normal. This phenotype suggested a potentially selective defect within the proximal TCR signaling pathway.

In short-term polyclonal cell lines from the three affected siblings, we observed no substantial increase in cytoplasmic free calcium ($[Ca^{2+}]_i$) after stimulation with either of two different CD3 mAbs [Fig. 1 and (*14*)]. This contrasted with increases in $[Ca^{2+}]_i$ in cells from both parents as well as in control cells enriched for $CD4^+$ T cells. Because mobilization of $[Ca^{2+}]_i$ in response to TCR stimulation results from tyrosine phosphorylation and activation of phospholipase C-γ1 (*1*), we analyzed the ability of the TCR to induce tyrosine phosphoproteins. In contrast to the responses of control cells, cross-linking of the patients' cells with CD3 mAbs alone or in combination with CD4 mAbs failed to induce substantial increases in tyrosine phosphoproteins [Fig. 2 and (*14*)]. Thus, the defect appears to reside within the early TCR-regulated PTK pathway.

Analysis of the PTKs implicated in TCR signaling demonstrated that this defect was associated with an absence of ZAP-70 (Fig. 3). No ZAP-70 protein was detected with two different antibodies to ZAP-70 (anti–ZAP-70): a mAb directed against the NH_2-terminal portion of ZAP-70 and a rabbit heteroserum directed against a peptide derived from sequences between the second SH2 and catalytic domains [Fig. 3 and (*14*)]. Normal levels of the Src family PTKs, Lck and Fyn, were present (Fig. 3), and comparable, albeit normally low, levels of the Syk PTK were detected in patient cells (*14*).

Whereas no protein was detected, normal levels of ZAP-70 mRNA from the patients' cells were detected (*14*) and could be analyzed. Two independent mutations were identified (Fig. 4). One consists of a C to A transition at position 1763, resulting in an S to R substitution at residue 518 (S, Ser; R, Arg) (Fig. 4A). Serine at position 518 resides in a highly conserved region of the catalytic domain of human ZAP-70 and is also present in murine ZAP-70, human Syk, and porcine Syk (*5*, *6*, *8*). The second mutation consists of a 9–base pair (bp) insertion, CTTGAG-

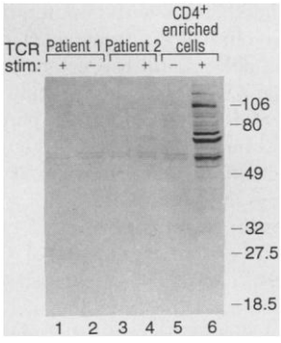

Fig. 2. Lack of induction of cellular tyrosine phosphoproteins in PBL lines from patients with SCID. PBL lines derived from patients 1 and 2, or CD8-depleted cells from a normal blood donor, as indicated, were incubated with a combination of biotinylated CD3 (Leu4) and CD4 (Leu3) mAbs and cross-linked with avidin for 2 min at 37°C (lanes 1, 4, and 6) (*19*). Unstimulated cells (lanes 2, 3, and 5) were treated with avidin only for 2 min at 37°C.

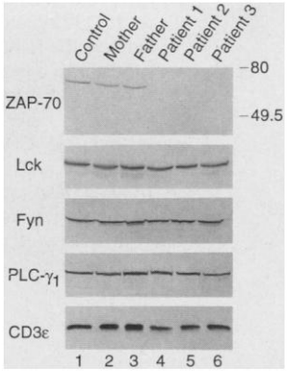

Fig. 3. Analysis of PTK expression. PBL lines from a normal donor (CD8-depleted; lane 1), the mother and father (lanes 2 and 3), and patients 1 to 3 (lanes 4 to 6) were lysed and analyzed by protein immunoblot with antibodies directed against ZAP-70, Lck, Fyn, PLC-γ1 and CD3ε (*20*). Molecular standards are indicated on the right (in kilodaltons).

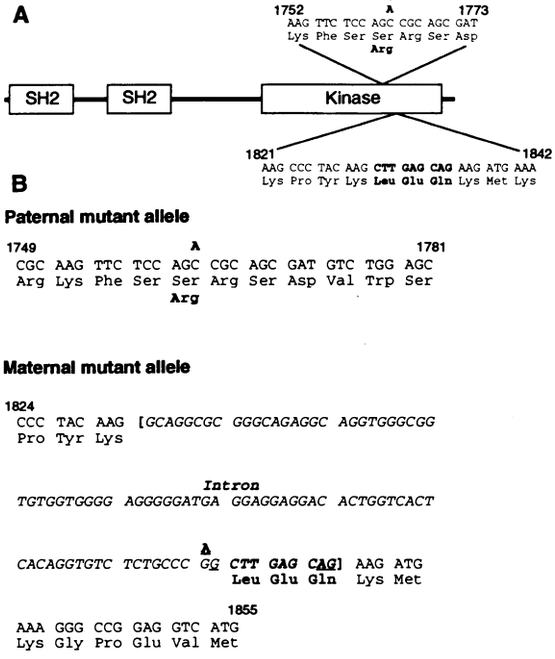

Fig. 4. DNA and deduced amino acid sequence of ZAP-70 defects in patient 1 and parents. (**A**) Schematic diagram of the two mutations found in ZAP-70 transcripts. The sequences in bold represent the mutated base pairs and the predicted amino acid changes. Seven different PCR cDNA subclones from four distinct amplifications from patient 1 were analyzed by standard dideoxy sequencing; two contained the C to A point mutation and five contained the 9-bp insertion (*21*). (**B**) Sequence of the paternal- and maternal-derived mutant ZAP-70 alleles. The underlined nucleotides represent the mutant and wild-type splice acceptor sites. The genomic analysis of patient 1 and parents was as follows: patient 1: of five clones, two contained the G to A intron mutation and three contained the C to A exon mutation; the mother: of six clones, three were wild type and three contained the intron mutation; and the father: of six clones, three were wild type and three contained the exon mutation.

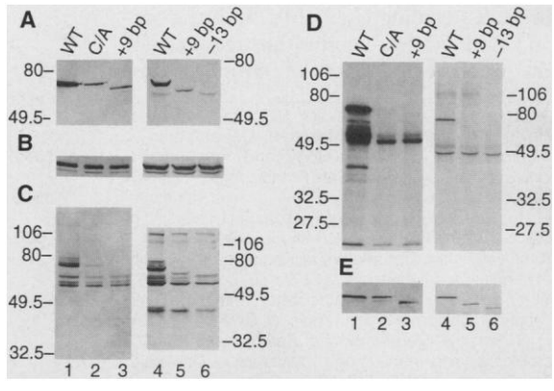

Fig. 5. Expression of mutant ZAP-70 molecules in COS-18 cells. Wild-type ZAP-70 and the mutant ZAP-70 cDNAs were cotransfected with human Lck into COS-18 cells that express a CD8-ζ chimera (*6*). Cells were harvested at 48 hours, and cell lysates were analyzed by protein immunoblot with (**A**) anti-ZAP-70, (**B**) anti-Lck, or (**C**) anti-phosphotyrosine mAbs (4G10). (**D**) ZAP-70 proteins, expressed in the absence of Lck, were also immunoprecipitated, analyzed for catalytic activity with the use of an in vitro kinase assay (*4*), and (**E**) analyzed for the amount of ZAP-70 present in each immunoprecipitate. Wild-type ZAP-70 is represented in lanes 1 and 4, the paternal mutant consisting of a C to A change is represented in lane 2, the maternal mutant consisting of a 9-bp insertion is represented in lanes 3 and 5, and the 13-bp deletion from the accompanying paper (*13*) is represented in lane 6.

CAG, and results in the addition of LEQ within the catalytic domain (L, Leu; E, Glu; Q, Gln). Analysis of partial genomic DNA clones derived from patient 1 demonstrated that these mutations are expressed on different chromosomes. The insertion is due to a G to A transition within an intron 9 bp upstream of the normal splice acceptor site (Fig. 4B). This mutation results in a more favored splice acceptor site, AGC, rather than AGA (*15*). Analysis of six partial genomic DNA clones derived from the parents demonstrated that they were each heterozygous, each parent expressing a wild-type and one mutant allele. The mutation giving rise to a new splice acceptor site was derived from the mother, and the C to A transition within the exon was derived from the father. Analysis of a rodent-human hybrid chromosome library and fluorescent in situ hybridization (FISH) demonstrated that ZAP-70 maps to chromosome 2q12 (*16*), consistent with the autosomal recessive nature of the defect.

We examined the significance of these mutations on protein expression and function. The mutant ZAP-70 molecules identified here, as well as a mutant resulting from a 13-bp deletion in an unrelated patient with similar T cell phenotypic and functional defects (*13*), were expressed in COS cells (Fig. 5). Expression of the three mutant alleles in COS cells resulted in lower levels of ZAP-70 protein despite higher levels of mRNA as compared with wild-type ZAP-70 [Fig. 5A and (*14*)]. The most notable decreases were observed with the 9-bp insertion and the 13-bp deletion. These were expressed as lower relative molecular weight forms of ZAP-70 and are likely to result from proteolytic cleavages of the mutant proteins that were not observed within the patient's cells. This may reflect more rapid turnover of these proteins in T cells as compared with their overexpression in COS cells. In vitro kinase assays revealed that all three mutant forms of ZAP-70 were catalytically inactive (Fig. 5, D and E). Whereas cotransfection of ZAP-70 with Lck or Fyn into COS cells that stably express a CD8-ζ chimera (COS-18) results in an induction of tyrosine phosphoproteins (*5, 10*), cotransfection of these ZAP-70 mutants with Lck resulted in fewer tyrosine phosphoproteins (Fig. 5C).

The distinct mutations in ZAP-70 in our patients and in the unrelated female patient described in the accompanying paper (*13*) are likely to represent the underlying molecular defect in this form of SCID phenotype. Taken together, these two unrelated families with this autosomal recessive form of SCID provide strong genetic evidence for the functional importance of ZAP-70. The induced association of ZAP-70 with the TCR subunits after receptor engagement previously implicated ZAP-70 in TCR signal transduction (*4–7, 9, 10*). Here, we suggest that the absence of functional ZAP-70 PTK gives rise to an immunodeficiency characterized by defects in TCR signal transduction. This is consistent with our studies in COS cells in which substitution of a kinase-inactive mutant for wild-type ZAP-70 that was coexpressed with a wild-type Src-family PTK resulted in the loss of tyrosine phosphorylation of cellular proteins (*10*).

The related PTK Syk has been implicated in TCR signaling (*6, 8*). Syk can associate with the stimulated TCR complex (*6*), raising the possibility that Syk may serve a redundant function in TCR signaling. ZAP-70 and Syk are expressed in both $CD4^+$ and $CD8^+$ T cells (*6*). However, the level of Syk protein in T cells is about one-twelfth that expressed in B cells (*6*). The inability of the SCID patient's $CD4^+$ T cells to signal suggests that this level of Syk expression is not sufficient to replace ZAP-70 function in TCR signaling in peripheral T cells.

TCR signal transduction plays an important role in T cell development. The ability of $CD4^+$ but not $CD8^+$ T cells to accumulate in the periphery of these SCID patients raises questions regarding the role of ZAP-70 in T cell development. ZAP-70 and Syk are expressed in all the major ($CD4^-CD8^-$, $CD4^+CD8^+$, and $CD4^+CD8^-$-$CD4^-CD8^+$) thymocyte populations, suggesting that both of these PTKs may play a role in TCR-regulated developmental decisions (*6*). Syk is present at fourfold higher levels in thymocytes than in peripheral T cells (*6*). In the absence of ZAP-70, Syk may play a preferential role in regulating the positive selection of $CD4^+$ T cells. Development of the $CD4^+$ lineage may be favored, perhaps as a result of the stronger association of Lck with CD4 relative to CD8 (*17*). Collectively, our studies and those of others (*22*) provide strong genetic evidence for the function of ZAP-70 in T cell development and activation through its involvement in TCR signal transduction.

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16. Chromosomal mapping of human ZAP-70 gene was done with a genomic DNA–based PCR-derived probe with oligonucleotides 1854 to 1868 and 1996 to 1971. A 1.4-kb fragment was obtained from human genomic DNA, and the ZAP-70 gene was mapped to chromosome 2 with a human-rodent somatic hybrid cell line library (Coriell, Camden, NJ). This 1.4-kb–derived PCR product was also used to isolate a genomic p1 clone for mapping by FISH [B. Trask *et al.*, *Am. J. Hum. Genet.* **48**, 1 (1991); A. Kallioniemi *et al.*, *Genomics*, in press].
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18. Peripheral blood lymphocytes (PBLs) from controls [which were also depleted of $CD8^+$ T cells with magnetic beads as described in (*6*)] and patients were grown in short-term cultures in the presence of RPMI-1640, 20% fetal calf serum, phorbol 12-myristate 13-acetate (PMA; 1 ng/ml), ionomycin (1 μM), and interleukin-2 (20 U/ml; Cetus, Emeryville, CA). Cells were washed and rested for 24 hours before assays. $[Ca^{2+}]_i$ was measured as previously described (*3, 9*).
19. Short-term polyclonal PBL lines as described in Fig. 1 were washed in phosphate-buffered saline (PBS), incubated with a combination of biotinylated Leu4 and Leu3 mAbs at 4°C for 20 min, washed with cold PBS to remove unbound mAb, and cross-linked with avidin (5 μg/ml) at 37°C for 2 min. Cell lysates were analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions and protein immunoblot with a phosphotyrosine mAb (4G10; Upstate Biological, Lake Placid, NY) as previously described (*5*).
20. Antibodies used included an Lck mAb, anti-Fyn, PLC-γ1 mAb (UBI), anti-CD3ε (DAKO), ZAP-70 mAb (*10*), and anti–ZAP-70 peptide (residues 326 to 341) (*5, 14*).
21. Total cellular RNA was harvested from cells with RNAzol (Tel-Test, Friendswood, TX). Seven complementary DNA (cDNA) clones from four different reverse transcriptase reactions using random primers for cDNA synthesis were used to initially identify mutations. Polymerase chain reaction (PCR) was performed with combinations of sense oligonucleotide primers encompassing base pairs 103 to 124 and 1119 to 1140 and antisense primers encompassing base pairs 1185 to 1205 and 2087 to 2108 within the ZAP-70 cDNA sequence (*5*). Genomic DNA was harvested and PCR performed with sense primers encompassing base pairs 1554 to 1572 and antisense primers encompassing base pairs 1854 to 1872. PCR products were subcloned into Bluescript KS(−) (Stratagene, San Diego, CA) and sequenced.
22. Similar findings were recently reported independently by E. Arpaia *et al.* [*Cell* **76**, 947 (1994)].

23. We thank W. Monafo and S. Polmar for their roles in the earlier descriptions of these SCID patients; R. Shapiro for assistance with the clinical management of the affected patients in this family; and J. Gray and the UCSF–Lawrence Berkeley Laboratory Resource for Molecular Cytogenetics for assistance in FISH mapping. We thank J. Bolen for providing Lck mAb and A. Veillette for providing anti-Fyn. A.C.C. is the recipient of an American Foundation for Clinical Research–Merck M.D.–Ph.D. postdoctoral award and an American College of Rheumatology Investigator award. Supported in part by National Institutes of Health grant AR-20684 (to A.C.C.) and GM39553 (to A.W.).

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