

BRIEF REPORT

## Inherited and Somatic *CD3 $\zeta$* Mutations in a Patient with T-Cell Deficiency

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### SUMMARY

A four-month-old boy with primary immunodeficiency was found to have a homozygous germ-line mutation of the gene encoding the *CD3 $\zeta$*  subunit of the T-cell receptor–CD3 complex. *CD3 $\zeta$*  is necessary for the development and function of T cells. Some of the patient's T cells had low levels of the T-cell receptor–CD3 complex and carried the Q70X mutation in both alleles of *CD3 $\zeta$* , whereas other T cells had normal levels of the complex and bore the Q70X mutation on only one allele of *CD3 $\zeta$* , plus one of three heterozygous somatic mutations of *CD3 $\zeta$*  on the other allele, allowing expression of poorly functional T-cell receptor–CD3 complexes.

THE ANTIGEN-RECEPTOR COMPLEX ON THE SURFACE OF T CELLS CONSISTS of a clonotypic heterodimer ( $\alpha$  and  $\beta$  chains in most T cells) and four invariant signaling subunits: *CD3 $\gamma$* , *CD3 $\delta$* , *CD3 $\epsilon$* , and *CD3 $\zeta$* . In mature T cells, the  $\zeta$  chain synthesis regulates the assembly of complete T-cell–receptor complexes and their expression on the cell surface in the steady state.<sup>1</sup> Complexes lacking the  $\zeta$  chain are assembled in the Golgi apparatus, but instead of moving on to the plasma membrane, as normal complexes do, they are rapidly shunted to lysosomes for degradation.<sup>2</sup> The *CD3 $\zeta$*  chain contains three copies of the immunoreceptor tyrosine-based activation motif (ITAM).<sup>3</sup> These activation motifs become phosphorylated on stimulation of the T-cell receptor by a ligand and then associate with the 70-kD zeta-associated protein (ZAP-70), a protein tyrosine kinase with a critical role in the initiation of T-cell signaling.<sup>4</sup> Hence, in addition to controlling the expression of T-cell receptor–CD3 complexes on the plasma membrane, *CD3 $\zeta$*  contributes to the mechanism of T-cell activation. Moreover, *CD3 $\zeta$*  also participates in intrathymic T-cell differentiation, which is arrested in mice lacking *CD3 $\zeta$* .<sup>5-8</sup>

Severe combined immunodeficiency is a heterogeneous group of diseases characterized by a profound block in T-cell development or function.<sup>9</sup> The absence of T cells causes defects in both cellular and humoral immunity. In patients lacking only mature T cells, mutations of the gene encoding the interleukin-7–receptor  $\alpha$  chain<sup>10</sup> and mutations of the *CD45* gene have also been reported.<sup>11,12</sup> A deficiency of B cells, natural killer cells, or both occurs in some cases of immunodeficiency. Cases of severe combined immunodeficiency with mutations in the *CD3 $\delta$*  or *CD3 $\epsilon$*  genes<sup>13,14</sup> demonstrate the essential role of *CD3 $\delta$*  and *CD3 $\epsilon$*  in T-cell differentiation. We describe a child with severe immunodeficiency associated with *CD3 $\zeta$*  deficiency.

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## CASE REPORT

The patient, a boy of Caribbean origin, was of unknown paternity. Two older siblings were healthy. He presented at the age of four months with erythroderma, protracted diarrhea, and pulmonary abscesses caused by *Pseudomonas aeruginosa*. During the next two years, he had recurrent episodes of herpes simplex virus infection of the mouth and skin, two episodes of oral and skin infections with *Candida albicans*, and two pulmonary infections, one of which was caused by *Streptococcus pneumoniae*. The patient's T-cell counts were very low, B-cell counts were normal, and there was eosinophilia (1000 cells per cubic millimeter) (Table 1). Neutrophil and platelet counts were normal. Lymphocytes of maternal origin could not be detected in the blood by fluorescence in situ hybridization, a finding that ruled out mother–infant graft-versus-host disease. Serum immunoglobulin levels were elevated (Table 1). IgM heterogeneity was restricted, and IgG autoantibodies against erythrocytes and neutrophils were detected. No antibodies were detected after immunization with tetanus toxoid, diphtheria toxoid, and poliovirus (Table 1). The patient received intravenous immune globulin therapy, antibiotics, and antifungal treatment. A haploidentical bone marrow transplantation, with the mother as the donor, was performed when the patient was 30 months old. The transplant resulted in sustained donor–recipient chimerism and correction of the immunodeficiency. Three years later, the patient is well and living at home. The study was approved by the institutional review board, and the patient's mother gave written informed consent for all investigations.

## METHODS

## ANALYSIS OF IMMUNE FUNCTION

Immunofluorescence analysis, assays for proliferation of peripheral-blood mononuclear cells, and studies of natural-killer-cell cytotoxicity were performed as previously described.<sup>15-18</sup> The Foxp3-specific monoclonal antibody (PCH101) was used according to the manufacturer's instructions. The serum immunoglobulin levels and the level of antibodies after immunization were determined by nephelometry and enzyme-linked immunosorbent assay for diphtheria and tetanus toxoids and polioviruses.

## IMMUNOPRECIPITATION AND WESTERN BLOT ANALYSIS

Cell lysis, cell biotinylation, and immunoprecipitation of proteins were performed as described.<sup>19</sup> Immunoprecipitates or lysates were subjected to sodium dodecyl sulfate–polyacrylamide-gel electrophoresis and blotted on polyvinylidene difluoride membranes (Millipore). Primary monoclonal antibodies specific for CD3 $\zeta$  (6B10.2, Santa Cruz), CD3 $\epsilon$  (UCHT1 and Apa1.1, provided by Dr. B. Alarcon of the University of Madrid), ZAP-70 (SC157, Santa Cruz; and clone 29, Transduction Laboratory), Fc $\epsilon$ RI $\gamma$  (a gift from Dr. C. Bonnerot<sup>20</sup>), antiphosphotyrosine (4G10, UBI), and tubulin (Ab-1, Oncogene) were used. Specific proteins were detected with appropriate horseradish peroxidase–conjugated second antibodies (Jackson ImmunoResearch Laboratories) and the ECL detection system (Amersham Pharmacia Biotech).

## MUTATIONS

Genomic DNA and RNA were extracted from peripheral-blood mononuclear cells, T cells (with either high or low levels of the T-cell receptor–CD3 complex) sorted by a fluorescence-activated cell sorter (FACS), B-cell lines transformed with Epstein–Barr virus (EBV), and fibroblasts, as previously described.<sup>21</sup> CD3 $\zeta$ -specific reverse-transcriptase polymerase chain reaction was performed according to a standard method and specific primers (see the Supplementary Appendix, available with the full text of this article at [www.nejm.org](http://www.nejm.org)). Polymerase-chain-reaction products either were sequenced directly, as previously described, or were cloned into Topo vectors (Invitrogen) and sequenced.

## RESULTS

## IMMUNOLOGIC INVESTIGATIONS

The patient's T-cell counts were low at the age of 10 months and gradually increased until the age of 2 years, but the levels remained below normal values (Table 1). The levels of natural killer cells and B-cell counts were normal (Table 1). Ninety percent of circulating T cells expressed low levels of CD3 $\epsilon$  and  $\alpha/\beta$  T-cell receptors and were designated as the subgroup with low levels of the T-cell receptor–CD3 complex; the remaining 10 percent of T cells had normal levels of the T-cell receptor–CD3 complex (Fig. 1A). We detected both CD4+ and CD8+ cells in the population that had low

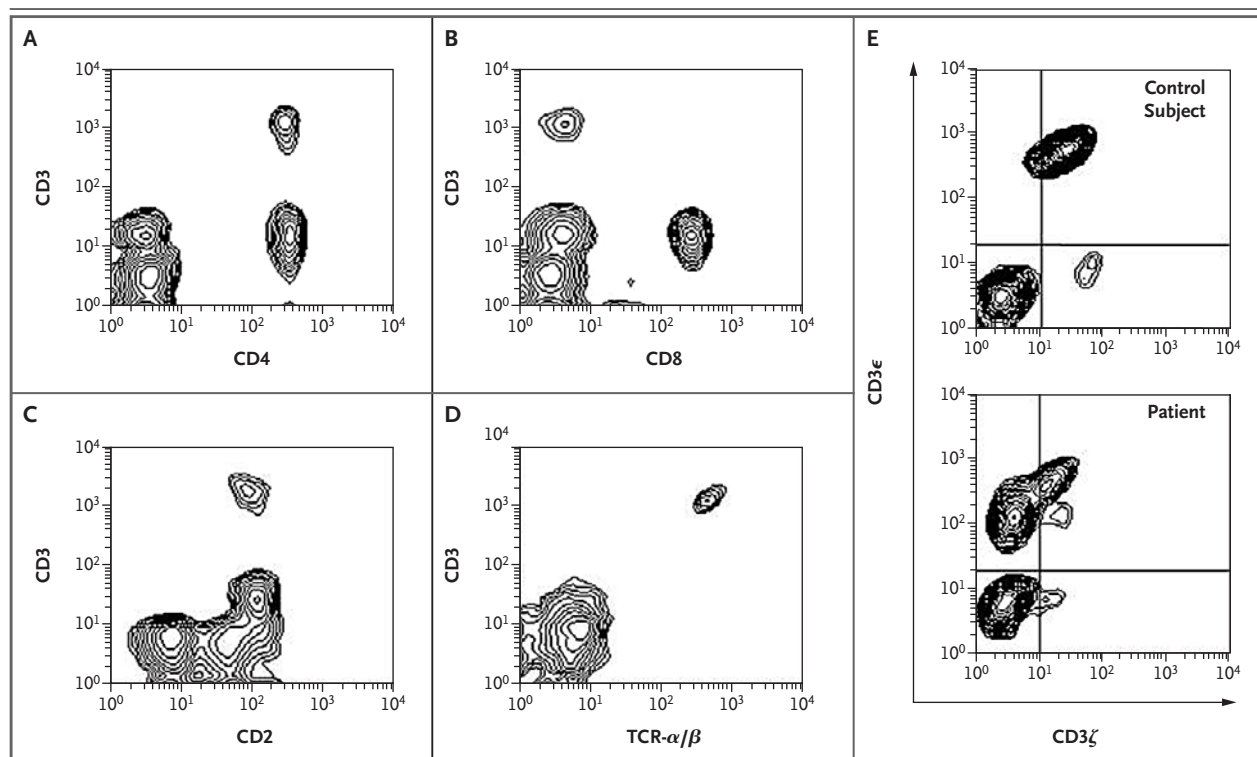
**Table 1. A Comparison of Immunologic Function in the Patient and in Controls.\***

Variable	Patient				Controls	
	10 Mo		26 Mo		Range	
	%	no./ $\mu$ l	%	no./ $\mu$ l	%	no./ $\mu$ l
Lymphocyte count	NA	1200	NA	2850	NA	4000–9000
Low levels of the T-cell receptor–CD3 complex	17	204	41	1168	60–85	2400–6000
Normal levels of the T-cell receptor–CD3 complex	4	48	6	171	60–85	2400–6000
CD4	21	252	32	912	35–50	1400–4000
CD8	1	12	17	484	20–35	500–2000
CD19	58	696	34	969	5–15	260–1400
CD16/CD56	13	156	17	484	3–10	200–900
<b>T-Cell Receptor–CD3 Complex</b>						
	Normal Level			Low Level		
Lymphocyte subgroup (%) <sup>†</sup>						
CD4	100			46		
CD8	0			54		
CD45RO	100			87		
CD4/CD45RA	0			15		
CD28	88			75		
CD27	77			71		
CD31	2			5		
Stimulus used in proliferation assay (counts/min $\times 10^{-3}$ )						
None (day 3)	1.1 $\pm$ 0.8			1.4 $\pm$ 1.1		
Phytohemagglutinin	9.3 $\pm$ 4			104 $\pm$ 51		
CD3 antibody (OKT3)	7.8 $\pm$ 9.6			52.8 $\pm$ 26.3		
CD3 antibody (50 ng/ml) and interleukin-2 (10 IU/ml)	34.0 $\ddagger$			62.8 $\pm$ 22.4		
10 <sup>-8</sup> M PMA and 10 <sup>-6</sup> M ionomycin	42.0 $\ddagger$			83 $\pm$ 35.0		
None (day 6)	0.5 $\pm$ 0.2			0.7 $\pm$ 0.5		
Tetanus toxoid	6.1 $\pm$ 4.9			34.4 $\pm$ 21.8		
Cytolytic activity of natural killer cells (% of chromium-51 released)						
None	60			57.4 $\pm$ 20.1		
Interferon- $\gamma$ (5000 IU/ml)	89			69.5 $\pm$ 19.6		
Serum immunoglobulin						
IgG (mg/dl)	1190–1500			310–570		
IgA (mg/dl)	170–230			26–82		
IgM (mg/dl)	300–430			40–113		
IgE (IU/ml)	1027–2900			<30		
Antibody production						
Tetanus toxoid (IU/ml)	<0.1			>0.1		
Diphtheria toxoid (IU/ml)	<0.1			>0.1		
Poliovirus I, II, III (titer $\times 10^{-1}$ )	0, 0, 0			>40		

\* Plus–minus values are means  $\pm$ SD. Control values are given for age-matched children. PMA denotes phorbol myristate acetate, and NA not applicable.

<sup>†</sup> Values were obtained when the patient was 10 months of age.

<sup>‡</sup> The analysis was performed once.



**Figure 1.** T-Cell Receptor–CD3 Expression by the Patient's T Cells.

Immunofluorescence analysis was performed on whole blood with the use of CD3 antibodies conjugated with phycoerythrin (PE) and antibodies against CD4 (Panel A), CD8 (Panel B), CD2 (Panel C), and  $\alpha/\beta$  T-cell receptor (TCR- $\alpha/\beta$ ) (Panel D) conjugated with fluorescein isothiocyanate (FITC). Immunofluorescence analysis was also performed on permeabilized whole-blood cells with the use of PE-conjugated CD3 $\epsilon$  antibodies and FITC-conjugated CD3 $\zeta$  antibodies (Panel E).

levels of the T-cell receptor–CD3 complex (Fig. 1A and Table 1). In contrast, almost all the T cells with normal levels of the T-cell receptor–CD3 complex were CD4+ (Fig. 1A and Table 1). Analysis by FACS of peripheral-blood mononuclear cells that were rendered permeable to allow entrance of analytical antibodies showed that the  $\zeta$  chain was present mainly in cells that strongly expressed CD3 $\epsilon$  (Fig. 1E). Most of the T cells, including all the lymphocytes with normal levels of the T-cell receptor–CD3 complex, displayed a phenotype of memory T cells (CD45RO+CD27+CD28+CD31–) (Table 1). No CD3+CD4+Foxp3+ regulatory T cells could be detected (data not shown).

In vitro, T-cell proliferation induced by phytohemagglutinin or CD3 antibodies was impaired, as compared with cells from control subjects, but the addition of interleukin-2 partially restored the response to CD3 antibodies (Table 1). The patient's T cells responded poorly to tetanus toxoid, as compared with T cells from an age-matched control subject. In contrast, sustained proliferation

of T cells was observed on stimulation with phorbol myristate acetate and ionomycin (Table 1). Since the two pharmacologic agents act synergistically to enhance T-cell activation independent of T-cell receptors, these results indicate that the proliferative defect in the patient's T cells was restricted to the initial steps of T-cell receptor–CD3 signaling. Normal cytotoxic activity of natural killer cells was observed with the K562 cell line (Table 1).

#### ANALYSIS OF THE T-CELL RECEPTOR–CD3 COMPLEX

With the use of appropriate antibodies, CD3 $\epsilon$  and CD3 $\zeta$  chains could not be precipitated from the plasma membranes of the patient's cells with low levels of the T-cell receptor–CD3 complex (Fig. 2A). In whole-cell lysates, the CD3 $\epsilon$  chain was detected, but the CD3 $\zeta$  chain was undetectable. In contrast, the CD3 $\zeta$  chain was detected by a Western blot analysis of plasma-membrane preparations of the patient's cells with normal levels of the T-cell receptor–CD3 complex but in somewhat

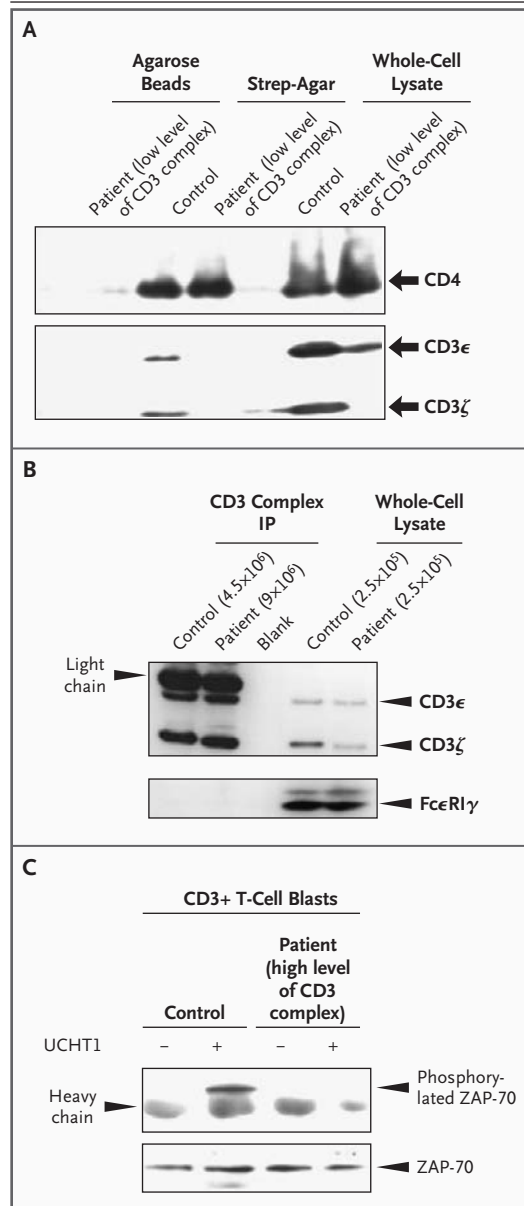
**Figure 2. Detection of CD3 $\epsilon$  and CD3 $\zeta$  Proteins in the Patient's T Cells with Either High or Low Levels of the T-Cell Receptor–CD3 Complex.**

In Panel A, purified T cells from a control donor and T cells with low levels of the T-cell receptor–CD3 complex from the patient were biotinylated and lysed. Biotinylated proteins were precipitated with streptavidin coupled to agarose (Strep-Agar). Streptavidin precipitates, nonspecific precipitates obtained with agarose beads, and total proteins from whole-cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to Western blotting with antibodies specific for CD4, CD3 $\epsilon$ , and CD3 $\zeta$ . In Panel B, phytohemagglutinin (PHA)-activated T-cell blasts with normal levels of the T-cell receptor–CD3 complex from a control and from the patient were sorted. After lysis, CD3 complexes were immunoprecipitated (IP) with an anti-CD3 $\epsilon$  monoclonal antibody. Immunoprecipitated proteins from  $4.5 \times 10^6$  blasts from a control subject and  $9 \times 10^6$  blasts from our patient and whole-cell lysates from  $2.5 \times 10^5$  blasts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and revealed by Western blot analysis with antibodies against CD3 $\epsilon$ , CD3 $\zeta$ , and Fc $\epsilon$ RI $\gamma$ . The light chain of the immunoprecipitating antibody is shown. In Panel C, phytohemagglutinin-activated T-cell blasts with normal levels of the T-cell receptor–CD3 complex from a control subject and the patient were sorted, left quiescent, or activated for three minutes with the anti-CD3 $\epsilon$  UCHT1 monoclonal antibody. After lysis, ZAP-70 was immunoprecipitated from  $7 \times 10^6$  blasts from a control subject and the patient. Immunoprecipitated ZAP-70 was revealed with the 4G10 phosphotyrosine monoclonal antibody to show the phosphorylated form of ZAP-70 or a ZAP-70 monoclonal antibody to illustrate the total level of expression of ZAP-70. The heavy chain of the immunoprecipitating antibody is shown.

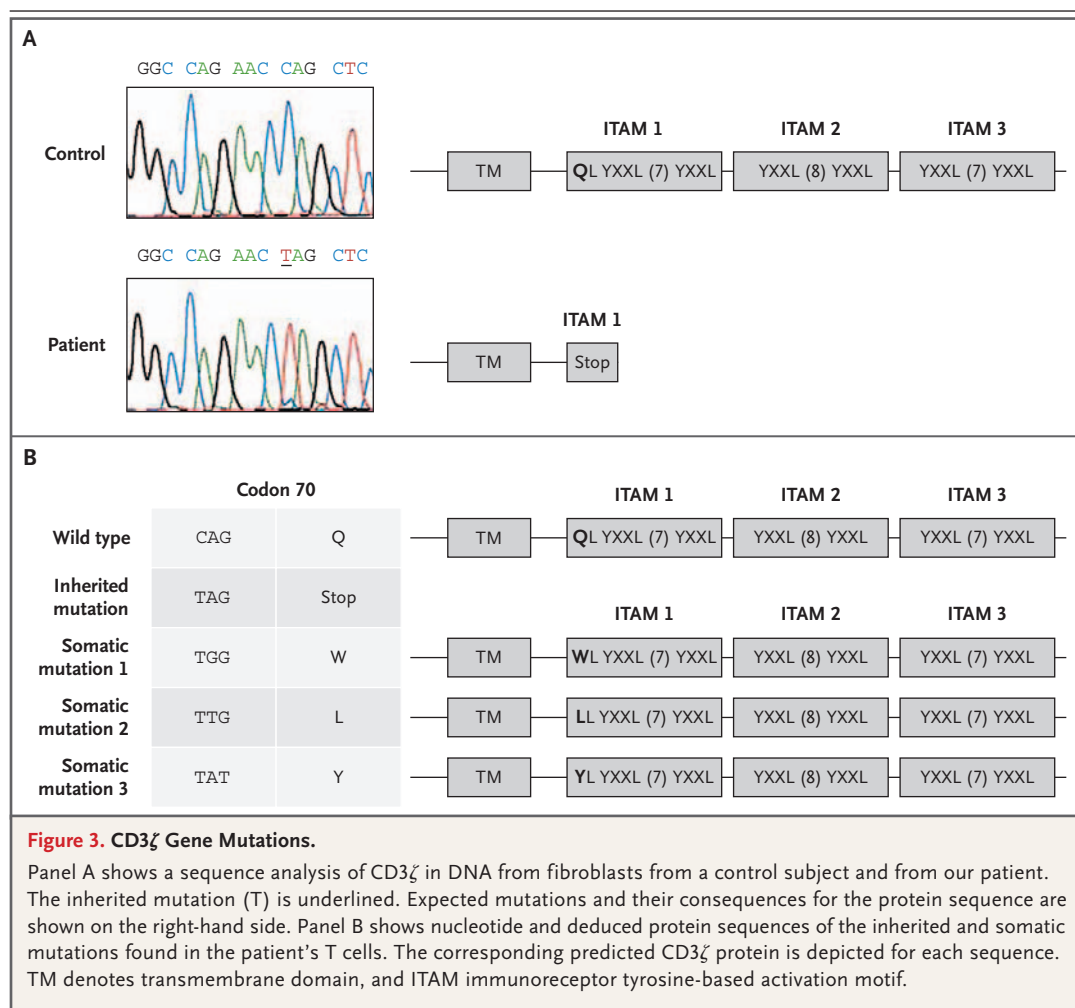
lower amounts than in control cells (Fig. 2B). Since the Fc $\epsilon$ RI $\gamma$  chain, which is involved in signaling the antigen receptor of B cells, is also found in T-cell receptor–CD3 complexes of intraepithelial T cells in mice,<sup>22</sup> we looked for this chain in the patient's T cells with normal levels of the T-cell receptor–CD3 complex. It was not found in plasma-membrane preparations obtained from the patient's T cells with normal levels of the T-cell receptor–CD3 complex but was present in normal amounts in the whole-cell lysate of these cells (Fig. 2B). ZAP-70 was present in normal amounts in the patient's CD3-activated T cells with normal levels of the T-cell receptor–CD3 complex but was not phosphorylated (Fig. 2C).

**ANALYSIS OF THE CD3 $\zeta$  GENE**

Given the defective expression of CD3 $\zeta$  by the patient's T cells with low levels of the T-cell recep-



tor–CD3 complex, we sequenced the CD3 $\zeta$  gene in DNA from lines of his EBV-transformed B cells and fibroblasts and from his mother's T cells. A homozygous C-to-T transition at nucleotide 207 of the coding sequence, leading to a nonsense mutation at position 70 (Q70X), was detected (Fig. 3A). This change was not found in 200 chromosomes from control subjects of the same ethnic origin as the patient and his mother. The premature stop codon of Q70X is located within the first ITAM domain, immediately upstream from the first YXXL motif, precluding expression of all ITAM motifs and thus any interaction with the tyrosine



kinase ZAP-70. A heterozygous Q70X mutation was identified in the mother's DNA, but no material was available from the father. Expression of the T-cell receptor-CD3 complex by the mother's T cells was normal (not shown), a finding consistent with inheritance of an autosomal recessive mutation of the *CD3 $\zeta$*  gene.

We detected the homozygous Q70X mutation in DNA from the patient's cells with low levels of the T-cell receptor-CD3 complex and in complementary DNA produced from messenger RNA (mRNA) from these cells. In contrast, sequence analysis of the *CD3 $\zeta$*  gene in DNA from the patient's T cells with normal levels of the T-cell receptor-CD3 complex showed that four sequences of the same mutated codon were present (Fig. 3B). One of these sequences matched the mutated TAG sequence in the Q70X mutation, whereas the other three, which were present in equal propor-

tions in total DNA from T cells with normal levels of the T-cell receptor-CD3 complex, had TGG, TTG, or TAT sequences at codon 70 (Fig. 3B). These sequences were not detected in DNA and RNA from the patient's fibroblasts, B cells, or T cells with low levels of the T-cell receptor-CD3 complex, or in DNA from more than 100 chromosomes from control subjects. Because the patient's T cells with normal levels of the T-cell receptor-CD3 complex were not of maternal origin, the TGG, TTG, and TAT variants must have arisen by somatic mutation of one of the germ-line Q70X alleles. As a result, the patient's subgroup of T cells with normal levels of the T-cell receptor-CD3 complex was a mixture of cells, each of which contained the inherited Q70X mutation on one allele and one of the three somatic mutations on the other allele. These somatic mutations would lead to full-length variants carrying a tryptophan (W), leucine (L), or tyrosine (Y) at position 70.

tophan (W), a leucine (L), or a tyrosine (Y) at position 70 (Q70W, Q70L, or Q70Y, respectively), which is consistent with our finding of a CD3 $\zeta$  chain that is close to its usual molecular weight at the plasma membrane in T cells with normal levels of the T-cell receptor–CD3 complex (Fig. 3B).

#### DISCUSSION

We describe a boy with greatly increased susceptibility to bacterial, viral, and fungal infections and a new type of T-cell immunodeficiency that was caused by a homozygous mutation (Q70X) of CD3 $\zeta$ . This subunit of the T-cell receptor–CD3 complex is essential for the expression of the complex and its signaling function. The mutant Q70X gene impaired formation of the complex on the plasma membrane and thus rendered the affected T cells incapable of activation through the antigen receptor. The impaired activation of T cells was probably related to a truncated CD3 $\zeta$  chain that was devoid of intracellular ITAM domains. These domains are necessary for the recruitment of the tyrosine kinase ZAP-70, which becomes activated by phosphorylation after recruitment to the complex. Phosphorylation of ZAP-70 allows signal transduction that culminates in T-cell activation. In 90 percent of the patient's T cells, the truncated CD3 $\zeta$  chain was detected in small amounts in the cytoplasm but not on the membrane. Defective membrane expression of the T-cell receptor–CD3 complex may result from instability of CD3 $\zeta$  mRNA, from the production of an unstable truncated protein, or from the role of the cytoplasmic tail of CD3 $\zeta$  in the expression and signaling function of T-cell receptor–CD3 complexes on the plasma membrane.<sup>23</sup> T-cell receptor–CD3 complexes lacking CD3 $\zeta$  have been shown to be shunted from the Golgi complex to the lysosomal pathway for degradation,<sup>2</sup> and in CD3 $\zeta$ -deficient mice, the expression of these complexes is profoundly impaired.<sup>5-8</sup> These mice also display a profound but incomplete block of T-cell development in the thymus,<sup>5-8</sup> and they have few CD4+ and CD8+ T cells in lymphoid organs.<sup>24</sup> Although in our patient the number of CD4+ and CD8+ T cells with low levels of the T-cell receptor–CD3 complex increased over time, the murine and human phenotypes generated by CD3 $\zeta$  deficiency appear to be similar with respect to peripheral T cells.

Other CD3 deficiencies variably affect the development and function of T cells. Null mutations

of the CD3 $\delta$  or CD3 $\epsilon$  gene lead to a complete absence of T cells.<sup>13,14</sup> CD3 $\gamma$  deficiency, however, causes a much milder immunodeficiency<sup>25</sup> than our patient had. The mosaic of phenotypes associated with deficiencies in the CD3 $\epsilon$ , CD3 $\delta$ , CD3 $\gamma$ , or CD3 $\zeta$  chain highlights the complexity of T-cell receptor–CD3 signaling patterns, which are poorly understood.

The germ-line mutation of CD3 $\zeta$  in our patient was associated with three somatic CD3 $\zeta$  mutations, all located at the mutated codon 70 of the CD3 $\zeta$  gene in the population with normal levels of the T-cell receptor–CD3 complex, which were obtained from 10 percent of the patient's T cells. These mutations apparently reversed the effect of the Q70X mutation and allowed the production of full-length CD3 $\zeta$  variants in T cells with normal levels of the T-cell receptor–CD3 complex. These variants stably bound to the other CD3 subunits, resulting in levels of T-cell receptor–CD3 complexes in the plasma membrane that were close to levels in normal T cells. The mechanism of the poor proliferation of the patient's T cells with normal levels of the T-cell receptor–CD3 complex, both in vitro and in vivo, is unclear. It could not have been due to interference by Fc $\epsilon$ R1 $\gamma$  chains, which were not found in T-cell receptor–CD3 complexes from the patient's T cells with normal levels of the T-cell receptor–CD3 complex. These T cells, which were CD4+, were not natural regulatory T cells,<sup>26</sup> because they did not express Foxp3, a transcription factor required for the development and function of such T cells. The lack of phosphorylated ZAP-70 protein in the patient's T cells with normal levels of the T-cell receptor–CD3 complex indicated that the CD3 $\zeta$  variants on the plasma membrane could not transduce an effective signal of T-cell activation. Possibly, mutations affecting the Q70 residue of the first ITAM motif of CD3 $\zeta$  impair the signaling function of this molecule.

The finding of somatic mutations of a germ-line mutation of the CD3 $\zeta$  gene recalls somatic mutations of germ-line mutations of the adenosine deaminase, interleukin-2 receptor  $\gamma$ c, recombination-activating gene 1, the Wiskott–Aldrich syndrome protein, and nuclear factor- $\kappa$ B essential modulator (NEMO, or IKK $\gamma$ ) genes. These somatic changes caused the mutant gene to revert to a wild-type gene or to a sequence compatible with expression of the corresponding protein.<sup>27-35</sup> The somatic mutations occurring in the initially mu-

tated codon of the *CD3 $\zeta$*  gene are probably not due to a mutational hot spot related to genomic instability, since these variants were not found in other cell types from our patient. The T cells with normal levels of the T-cell receptor-CD3 complex were polyclonal, and each of the three somatic variants of *CD3 $\zeta$*  was found in different populations of T cells, each with a distinctive rearrangement of *V $\beta$*  genes (data not shown). This result indicates that the somatic mutations in *CD3 $\zeta$*  probably occurred before the VDJ recombination process in T-cell progenitors.

In summary, we describe a form of T-cell immunodeficiency related to a recessive mutation

of the *CD3 $\zeta$*  gene. This observation adds to the reported variability in deficiencies of the various CD3 subunits. The characterization of somatic mutations partially correcting the *CD3 $\zeta$*  deficiency provides an example of the modulation of T-cell immunodeficiency by somatic mutations and of the selection of clones with such mutations.

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