



Universiteit
Leiden
The Netherlands

The impact of single amino acid substitutions in CD3gamma on the CD3epsilongamma interaction and T-cell receptor-CD3 complex formation

Thomassen, E.A.J.; Dekking, E.H.A.; Thompson, A.; Franken, C.L.M.C.; Sanal, Ö; Abrahams, J.P.; ... ; Koning, F.

Citation

Thomassen, E. A. J., Dekking, E. H. A., Thompson, A., Franken, C. L. M. C., Sanal, Ö., Abrahams, J. P., ... Koning, F. (2006). The impact of single amino acid substitutions in CD3gamma on the CD3epsilongamma interaction and T-cell receptor-CD3 complex formation. *Human Immunology*, 67(8), 579-588. doi:10.1016/j.humimm.2006.04.015

Version: Publisher's Version

License: [Licensed under Article 25fa Copyright Act/Law \(Amendment Taverne\)](#)

Downloaded from: <https://hdl.handle.net/1887/3620877>

Note: To cite this publication please use the final published version (if applicable).

The Impact of Single Amino Acid Substitutions in CD3 γ on the CD3 $\epsilon\gamma$ Interaction and T-Cell Receptor–CD3 Complex Formation

E.A.J. Thomassen, E.H.A. Dekking, A. Thompson,
K.L. Franken, Ö. Sanal, J.P. Abrahams,
M.J.D. van Tol, and F. Koning

ABSTRACT: The human T-cell receptor–CD3 complex consists of at least eight polypeptide chains; CD3 $\gamma\epsilon$ - and $\delta\epsilon$ -dimers associate with the disulfide linked $\alpha\beta$ - and $\zeta\zeta$ -dimers to form a functional receptor complex. The exact structure of this complex is still unknown. We now have examined the interaction between CD3 γ and CD3 ϵ in human T-cells. For this purpose, we have generated site-directed mutants of CD3 γ that were introduced in human T-cells defective in CD3 γ expression. Cell-surface and intracellular expression of the introduced CD3 γ chains was determined, as was the association with CD3 δ , CD3 ϵ , and the T-cell receptor. Although the introduction of wild type CD3 γ and CD3 γ (78Y-F) fully restored T-cell receptor assembly and expression, the introduction of CD3 γ (82C-

S), CD3 γ (85C-S), and CD3 γ (76Q-E) all resulted in an impaired association between CD3 γ and CD3 ϵ and a lack of cell-surface expressed CD3 γ . Finally, the introduction of CD3 γ (76Q-L) and CD3 γ (78Y-A) restored the expression of TCR–CD3 $\delta\epsilon\gamma\epsilon\zeta_2$ complexes, although the association between CD3 γ and CD3 ϵ was impaired. These results indicate that several amino acids in CD3 γ are essential for an optimal association between CD3 γ and CD3 ϵ and the assembly of a cell-surface expressed TCR–CD3 $\delta\epsilon\gamma\epsilon\zeta_2$ complex. *Human Immunology* 67, 579–588 (2006). © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: CD3 γ deficiency; dimer interaction

ABBREVIATIONS

BSA bovine serum albumin
CD clusters of differentiation
DNA deoxyribonucleic acid
EDTA ethylenediaminetetra-acetic acid
Fab fragment antigen-binding
FACS fluorescence-activated cell sorter
FCS fetal calf serum
FITC fluorescein isothiocyanate
GFP green fluorescent protein
HAS human serum albumin
Ig immunoglobulin
IRES internal ribosomal entry site
ITAM immunoreceptor tyrosine-based activation motif

mCi millicurie
MHC major histocompatibility complex
MIM Mendelian Inheritance in Man
NMR nuclear magnetic resonance
NP40 Nonident-40
PAGE polyacrylamide gel electrophoresis
PAS protein A sepharose
PBMC peripheral blood mononuclear cells
PBS phosphate-buffered saline
PE phycoerythrin
rIL recombinant interleukin
SDS sodium dodecylsulphate
TCR T-cell receptor

INTRODUCTION

The majority of human T-cells express a clonotypic $\alpha\beta$ T-cell receptor (TCR) heterodimer. For the full function

of the receptor, the disulfide-linked $\alpha\beta$ -chains associate with CD3 δ , CD3 ϵ , CD3 γ , and ζ . These latter form noncovalently linked $\delta\epsilon$ and $\epsilon\gamma$ heterodimers and disulfide-linked ζ – ζ homodimers. While each TCR–CD3

From the Department of Immunobematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands (E.A.J.T., E.H.A.D., A.T., K.L.F., F.K.); Biophysical Structural Chemistry, Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands (E.A.J.T., J.P.A.); Hacettepe University Children's Hospital, Ankara, Turkey (Ö.S.); and the Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands (M.J.D.T.).

Address reprint requests to: F. Koning, Department of Immunobematology and Bloodtransfusion, E3-Q, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands; Tel: +31 (0) 71 5263800; Fax: +31 (0) 71 5216751; E-mail: F.Koning@lumc.nl.

complex therefore consists of a minimum of eight polypeptides, the exact stoichiometry of the complex is still under discussion [1–4]. The TCR $\alpha\beta$, which has a Fab-like structure, is responsible for the recognition of a specific antigen bound to MHC molecules. Subsequently, the CD3 and ζ components mediate signal transduction and intracellular activation [5–8]. CD3 δ , CD3 ϵ , and CD3 γ all have a large extracellular immunoglobulin (Ig)-like domain, a membrane proximal stalk region, a transmembrane helix, and an intracellular immunoreceptor tyrosine-based activation motifs (ITAM)-containing domain. This is in contrast to ζ , which has a small extracellular domain and a large intracellular domain with three ITAMs. The CD3 components are not only required for transduction of the signal across the cell membrane but also for the expression of the TCR heterodimers at the surface of T cells. In the absence of one of the CD3 chains—for example, because of a deleterious mutation—a reduced number of T-cell receptors is present at the cell surface [9–11].

CD3 deficiencies in humans are very rare autosomal disorders. In 1986, Regueiro *et al.* [12] reported a human CD3 γ deficiency [13]. This was the first primary T-cell receptor immunodeficiency in humans for which the genetic basis could be elucidated, Mendelian Inheritance in Man (MIM) number 186740. In 1990 another deficiency (MIM 186830) was reported by Thoenes *et al.*, which was later described as a partial CD3 ϵ deficiency [14–16]. Recently, de Saint Basile *et al.* [17] investigated the molecular mechanism underlying a severe combined immunodeficiency characterized by the selective absence of T cells and described as a complete CD3 ϵ deficiency. In 2003, Dadi *et al.* [18] studied three cases of CD3 δ deficiency. In total, three cases of human CD3 γ deficiency have been published in the mutation database; two are Spanish siblings, and the third patient is a Turkish male with an A to T mutation at position 242 in his CD3 γ DNA, which changes a lysine codon (AAA) to an early-stop codon (TAA) (not shown [19]).

In 2001, Sun *et al.* [20] described mechanisms contributing to T-cell receptor signaling and assembly, as revealed by the structure of the ectodomain of the murine CD3 $\epsilon\gamma$ heterodimer. Mutational analysis of CD3 ϵ , focusing on the binding interface between CD3 ϵ and CD3 γ , implicated several amino acids in CD3 ϵ and CD3 γ as being important for the domain–domain interaction. In this analysis, combinations of mutations in CD3 ϵ were found to be required for strong effects on the association between CD3 ϵ and CD3 γ . More recently, the structure of the human CD3 $\epsilon\gamma$ heterodimer in complex with the orthoclone K T-cell receptor 3 (OKT3) monoclonal antibody was elucidated [21]. Small differences were observed between the human and the murine structure, but whether these are caused by the different ori-

gins of the heterodimers (human CD3 ϵ and CD3 γ share 41% and 43% sequence homology to their murine counterparts, respectively) or by the different methods employed (crystallography versus nuclear magnetic resonance) is unknown. Both the solution structure of the CD3 $\epsilon\delta$ ectodomain [22] and the crystal structure of the CD3 $\epsilon\delta$ heterodimer complexed to the OKT3 monoclonal antibody were determined [23] in 2004. Despite all of these structures, the precise stoichiometry of the TCR-CD3 complex still is the subject of major discussions in the field. In one of the most recent studies, Call *et al.* [24] suggest that the $\alpha\beta$ TCR-CD3 complex is composed of one copy of the $\alpha\beta$ TCR, CD3 $\delta\epsilon$, CD3 $\gamma\epsilon$ and $\zeta\zeta$ dimers. The assembly of this TCR-CD3 complex was found to follow discrete steps in which the transmembrane region of CD3 $\delta\epsilon$, CD3 $\gamma\epsilon$ and $\zeta\zeta$ dimers play an important role [25]. They showed that each basic TCR residue in the helical transmembrane-spanning domain is required for assembly with a particular signaling dimer, thereby forming a three-helical structure. Two lysine residues are located in the middle of the transmembrane region of $\alpha\beta$ TCR and serve as critical points for assembly with CD3 $\delta\epsilon$ and CD3 $\gamma\epsilon$ dimers, whereas the $\zeta\zeta$ dimer associates with the arginine residue present in the α TCR.

In the present study we have further analyzed the binding interface of the CD3 $\epsilon\gamma$ dimer in human T-cells by introducing mutations in CD3 γ and determining the ability of such mutated CD3 γ chains to form CD3 $\gamma\epsilon$ dimers and participate in the formation of cell-surface-expressed TCR-CD3 complexes. The results demonstrate that single amino acid alterations in CD3 γ can have a significant effect on the composition of the cell-surface-expressed TCR-CD3 complex and provide further evidence that $\alpha\beta$ TCR $\delta\epsilon\delta\epsilon\zeta\zeta$ complexes can be formed even when the association of CD3 $\epsilon\gamma$ -dimers is impaired.

MATERIALS AND METHODS

Isolation of Peripheral Blood Mononuclear Cells and Establishment of T-Cell Lines

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood obtained from the patient, his parents, and a healthy older brother; Ficoll density gradient centrifugation was used. For the generation of T-cell lines, PBMCs (5×10^6) were cultured in the presence of allogeneic, irradiated (3000 rad) PBMCs (10×10^6), 50 U/mL recombinant interleukin-2 (rIL2) (Chiron, Amsterdam, The Netherlands), and 5 μ g/mL phytohemagglutinin (Murex Diagnostics, Dartford, UK) in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen, Breda, The Netherlands) supplemented with 2 mM glutamine (Invitrogen, Breda, The Netherlands), 100 U/mL penicillin (Invitrogen, Breda, The Netherlands), 100 μ g/mL streptomycin (Invitrogen, Breda, The

Netherlands), and 10% fetal calf serum (Greiner, Alphen a/d Rijn, The Netherlands). Twice a week, half of the supernatant was replaced by fresh medium containing 50 U/mL rIL2, and once in a period of 3 weeks allogeneic, irradiated PMBCs were added. After a culture period of 2 months, the dose of rIL2 was increased to 100 U/mL, and cells were harvested 1 month later.

Derivation of Mutants and Construction of Retroviral Vectors

A bicistronic vector, LZRS β BMN-linker-IRES-GFP, was used as described by Heemskerk *et al.* [26], with the gene of interest linked to a downstream internal ribosomal entry site (IRES) and a marker gene (Green fluorescent protein [GFP]) that allows independent translation of the products of both genes in the transduced cells. The vector was adapted for the gateway cloning system (Invitrogen, Breda, The Netherlands). Site-directed mutagenesis was performed using the polymerase chain reaction according to Landt [27] using a CD3 γ cDNA clone [28] as the template with custom-made primers. The mutations were made at AA position 78, tyrosine (TAT) was changed to a phenylalanine (TTT) and an alanine (GCT), at AA position 76 where the glutamine (CAA) was mutated to a glutamate (GAA) or a leucine (CTA), and at AA positions 82 and 85 where the cysteines (TGT) were mutated to serines (TCT). The resulting PCR products were cloned into the vector, and clones were confirmed by automated fluorescent sequencing.

Generation of Retroviral Supernatant and Retroviral Transduction

Phoenix cells, a 293T-based amphotropic retroviral packaging cell line [29], were transfected via the calcium phosphate precipitation method (Life Technologies, Gaithersburg, MD, USA), and after 2 days 2 μ g/mL puromycin (Clontech, Palo Alto, CA, USA) were added. At 10 to 14 days after transfection, 6×10^6 cells per 10-cm² petri dish were plated (Beckton Dickinson, Meylan, France) in 10 mL Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah, USA) without puromycin. After 24 hours the medium was refreshed, and on the following day retroviral supernatant was harvested, which was frozen at -70°C . Non-tissue-culture-treated plates (Beckton Dickinson, Meylan, France) were coated with retromectin (Takara, Shiga, Japan) for 2 hours at room temperature. The coated wells then were blocked with 2% human serum albumin (CLB, Amsterdam, The Netherlands). T cells were added, and after 30 minutes culture medium was removed, and thawed retroviral supernatant was supplemented. Viral supernatant together with cells were incubated overnight, followed by washed, and finally transferred to cell culture bottles in

normal tissue culture medium. After 3 days, transduced cells were sorted by a fluorescence-activated cell sorter (FACS) on an FACSVantage (Becton Dickinson, Mountain View, CA, USA) on the basis of high-level GFP expression. After several weeks of culture, the GFP⁺ populations of the several mutant T-cells were used for the experiments.

Cell Culturing

The patient's cells and the mutant cells were cultured in Iscove's modified Dulbecco's medium (Gibco BRL, Breda, The Netherlands) supplemented with 3 millimolar (mM) glutamine, 10% normal human serum, and 100 U rIL2/mL. Medium was refreshed every 3 days, and the cells were restimulated every 10 to 14 days with 100 U rIL2, 1 μ g/mL phytohemagglutinin, and 10×10^6 mL irradiated (2500 rad) PMBCs as feeder cells.

Antibodies

The antibodies against CD3 δ , CD3 ϵ , and CD3 γ were all rabbit antibodies and directed against peptides corresponding to the carboxy termini of the human CD3 chains. The anti-CD3 ζ serum was raised against the predicted N-terminus of the human ζ chain, as has been described before [30]. The anti-HLA class-I antibody (W6/32) [31], which was used as a control, is a mouse monoclonal antibody. The $\alpha\beta$ TCR-specific antibody used was BMA031 [32].

Cell Surface Iodination and Metabolic Labeling

For cell-surface iodination, 10×10^6 cells were washed three times in phosphate-buffered saline (PBS) and resuspended in 30 μ L lactoperoxidase (250 U/mL; Sigma, Zwijndrecht, The Netherlands) solution. One mCi Na¹²⁵I (NEN) was added to the cells followed by the addition of 10 μ L 0.05% H₂O₂/PBS (Sigma, Zwijndrecht, The Netherlands) with 5-minute intervals. After the labeling was stopped and the free iodine was removed by washing the cells three times with 500 μ L 2 mM KI/PBS, the cells were solubilized in 750 μ L lysis buffer containing 0.5% NP40 (Sigma, Zwijndrecht, The Netherlands) and protease inhibitors (Complete, EDTA-free tablets, Roche Diagnostics, Almere, The Netherlands). After 30 minutes incubation on ice, the lysates were centrifuged for 15 minutes ($13000 \times g$) at 4°C . For ³⁵S metabolic labeling, 7×10^6 vital cells were washed with 10 mL RPMI without methionine and cysteine, centrifuged, and resuspended in 10 mL of the same medium. Cells were incubated for 30 minutes at 37°C /5% CO₂ and centrifuged, and the resulting pellets were resuspended in 10 mL RPMI without methionine/cysteine, 100 U/mL IL2, and 1 mCi ³⁵S-methionine/cysteine. After 4 hours of incubation at 37°C /5% CO₂, cells were centrifuged and washed twice with PBS. The cells were

solubilized in 750 μ L lysis buffer containing 0.5% NP40 or 1% digitonine and protease inhibitors. After 30 minutes of incubation on ice, the lysates were centrifuged for 15 minutes ($13000 \times g$) at 4°C.

Immunoprecipitation and SDS-PAGE Analysis

The lysates, after labeling with 125 I or 35 S, were pre-cleared by adding 100 μ L protein A sepharose CL4B (PAS beads, Amersham Pharmacia Biotech, Roosendaal, The Netherlands), gently shaken at room temperature, followed by the addition of 75 μ L normal rabbit serum and shaken at room temperature for 1 hour. After the removal of the beads, specific immunoprecipitations with CD3 δ , CD3 ϵ , CD3 ζ , or CD3 γ antibodies, normal rabbit serum, or anti-HLA class I were performed by adding 5 μ L antiserum (2 μ L for anti-HLA class I) to 100 μ L pre-cleared lysate (1D SDS-PAGE) or 200 μ L pre-cleared lysate (2D SDS-PAGE) for 60 minutes at room temperature. The PAS beads were washed four times with 500 μ L lysis buffer and resuspended in 100 μ L 1 $\times$ sample loading buffer (20 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue, 1% SDS, 10% glycerol, 1% β -mercaptoethanol) and analyzed on a reducing 12% SDS-PAGE. For 2-dimensional SDS-PAGE, the PAS beads were resuspended in loading buffer without 2-mercaptoethanol and loaded onto the first nonreducing 12% cylindrical gel. After the first dimension, the gel was incubated for 10 minutes in Laemmli buffer with β -mercaptoethanol and placed on top of the reducing second dimension 12% SDS-PAGE gel. After drying of the gels, autoradiography was performed at -80°C using Kodax or Fuji scientific imaging films.

Fluorescence-Activated Cell Sorter (FACS) Analysis

For single stainings, cells were washed with PBS/0.5% BSA and labeled with anti-CD3-PE (Becton Dickinson, Erembodegem, Belgium) at 4°C for 1 hour. This was followed by washing with PBS/0.5% bovine serum albumin (BSA) and fixation with 0.5% paraformaldehyde. In the case of CD3/CD4 and CD3/CD8 double stainings, the cells were first incubated with anti-CD3-PE, washed with PBS/0.5% BSA, and labeled with anti-CD4-FITC or anti-CD8-FITC antibody thereafter (Becton Dickinson, Erembodegem, Belgium). For CD3/ $\alpha\beta$ -TCR double stainings, cells were first incubated with anti- $\alpha\beta$ -TCR antibody (BMA031, Immunotech, Marseilles, France) for 1 hour at 4°C and then incubated with goat anti-mouse-fluorescein isothiocyanate for 1 hour. Subsequently, the cells were washed and incubated with PBS supplemented with 1% mouse serum before staining with anti-CD3-PE. Finally, the cells were washed with PBS/0.5% BSA and fixed.

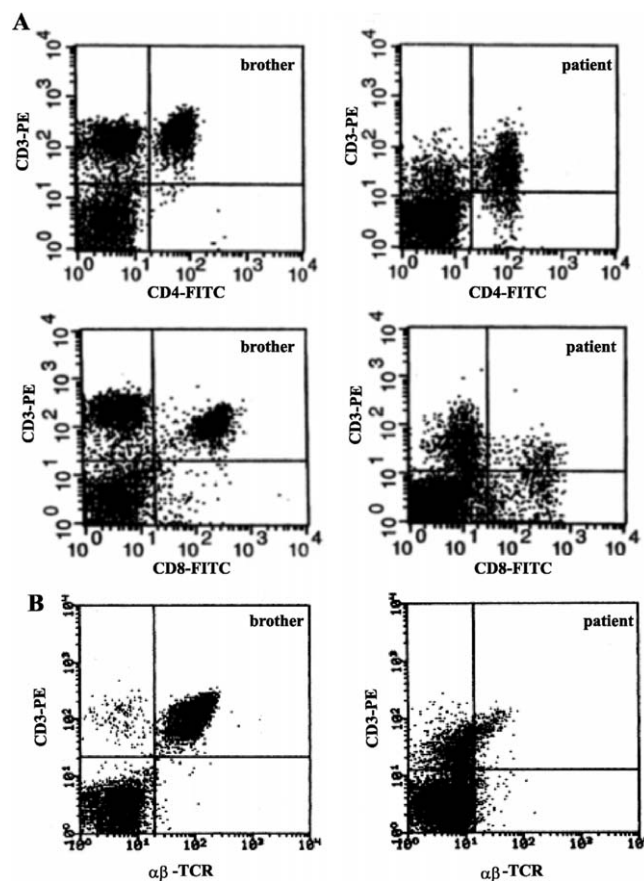


FIGURE 1 (A) FACS analysis of double-stained PBMCs of the patient (right panel) and healthy brother (left panel). The upper panel shows the CD3/CD4 stainings, and the lower panel shows the CD3/CD8 staining. (B) FACS analysis of T-cell lines established from PBMCs of the patient and brother. The left panel shows the CD3/ $\alpha\beta$ -TCR staining of the brother, and the right panel shows the CD3/ $\alpha\beta$ -TCR analysis for the CD3 γ -deficient patient. Whereas 96% of the T cells of the brother were $\alpha\beta\text{TCR}^+$, only 2% of the patient's cells were $\alpha\beta\text{TCR}^+$.

RESULTS

Characterization of TCR-CD3 Expression on T Cells from a Patient with a CD3 γ Deficiency

A male patient was diagnosed with CD3 γ deficiency at the age of 4 [33]. His parents are first cousins. An AAA-to-TAA mutation at position 242 of CD3 γ leads to an early-stop codon (not shown [19]). Because of this mutation, the remaining transcript would encode a 46-amino-acid-long protein lacking both the transmembrane region and the cytoplasmic domain. Peripheral blood mononuclear cells of the patient and his healthy brother were subjected to FACS analysis (Figure 1A). A CD3-PE/CD4-FITC and CD3-PE/CD8-FITC analysis indicated that the patient had diminished levels of CD3 expression on the cell surface of both T-cell populations.

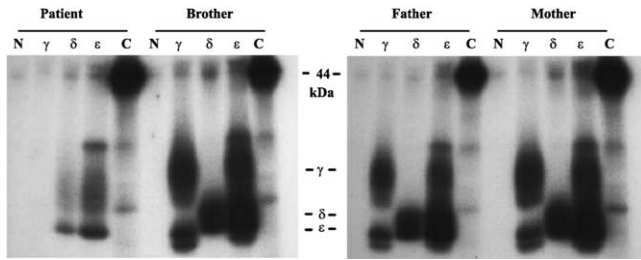


FIGURE 2 SDS-PAGE analyses of immunoprecipitates obtained from NP40 lysates after cell-surface iodination of T-cell lines of patient, brother, father, and mother. Antisera used were normal rabbit serum as negative control (N), anti-CD3 δ (δ), anti-CD3 ϵ (ϵ), and anti-CD3 γ (γ), and as reference (C) anti-HLA class I is shown. (Only heavy chain is shown here, 44 kDa.) The positions of the different chains are indicated in the middle.

The TCR-CD3 expression in the CD3 γ -deficient patient was found to be lower on CD8⁺ T-cells as compared with that on CD4⁺ T-cells. This phenomenon also was observed in other CD3 γ -deficient patients [34]. Next, T-cell lines were established from the PBMCs of the patient, his brother, and his parents. After 14 days of culture, an FACS analysis demonstrated that more than 96% of the T cells of the healthy brother expressed an $\alpha\beta$ TCR on the cell surface, whereas only about 2% of the patient's cells were $\alpha\beta$ TCR⁺ (Figure 1B). To investigate the cell-surface expression of the individual CD3 γ -, δ -, and ϵ -chains, the cell lines were labeled with ¹²⁵I and lysed in NP40 lysis buffer. Subsequently, immunoprecipitations were carried out with CD3 δ -, CD3 ϵ -, and CD3 γ -specific antibodies, followed by 1-dimensional SDS-PAGE analysis (Figure 2). In NP40 lysis buffer the TCR-CD3 complex dissociates into TCR $\alpha\beta$, ζ , CD3 $\gamma\epsilon$, and CD3 $\delta\epsilon$ dimers [36]. Consequently, CD3 γ and CD3 ϵ are present in CD3 γ immunoprecipitates, CD3 δ and CD3 ϵ in CD3 δ immunoprecipitates, and all three CD3 chains in CD3 ϵ immunoprecipitates. Although in the lysates of the cell lines from the patient's healthy brother and his parents CD3 δ , CD3 ϵ , and CD3 γ were present and associated as expected, the lysate of the cell line of the CD3 γ -deficient patient contained very little CD3 δ and CD3 ϵ , and CD3 γ was undetectable (Figure 2). Also, the mobility of CD3 δ appears different in the patient's T cells as compared with those of the healthy relatives (Figure 2). This may be because of abnormal glycosylation of CD3 δ as described [34, 35].

To verify the presence of TCR-CD3 components intracellularly, the T-cell lines were labeled with ³⁵S-methionine/cysteine, and after lysis in NP40 lysis buffer, specific immunoprecipitations were carried out with CD3 γ -, CD3 δ -, CD3 ϵ -, and CD3 ζ -specific antibodies, followed by SDS-PAGE analysis. This demonstrated (Figure 3) that the CD3 γ , CD3 δ , CD3 ϵ , and CD3 ζ

chains were present intracellularly in the T-cell lines obtained from the parents and the healthy brother, while the T cells of the patient contained CD3 δ , CD3 ϵ , and CD3 ζ chains at levels comparable with the healthy controls but no CD3 γ . Both parents are heterozygous for the CD3 γ mutation, which may explain the apparently diminished level of CD3 γ as compared with their healthy son (Figure 3). The presence of CD3 γ in both parents, however, is proven by the presence of CD3 ϵ in the immunoprecipitates of NP40 lysis performed with an antibody against CD3 γ . A 2-dimensional, nonreducing/reducing SDS-PAGE analysis of the ζ immunoprecipitate confirmed the normal expression of a ζ dimer (Figure 4). To determine the expression of the covalently linked TCR $\alpha\beta$ chains, immunoprecipitates were obtained with the anti-CD3 ϵ antibody from digitonin lysates of the metabolically labeled cells from the patient and his healthy brother. (Digitonin is known to preserve the subunit interactions between the TCR and CD3 complexes. [30]) These immunoprecipitates were subjected

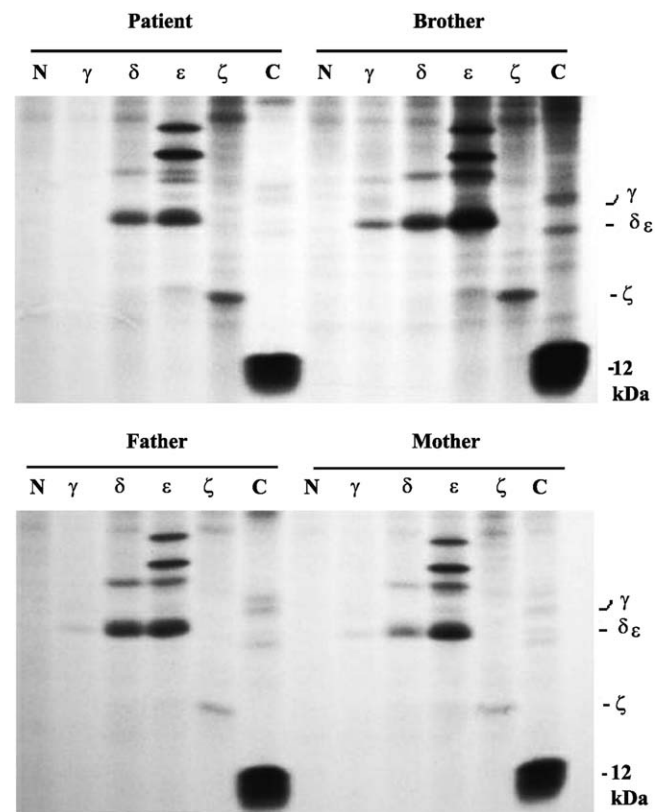


FIGURE 3 SDS-PAGE analysis of immunoprecipitates obtained from NP40 lysates after metabolic labeling of T-cell lines of patient, brother, father, and mother. Antisera used were normal rabbit serum as negative control (N), anti-CD3 δ (δ), anti-CD3 ϵ (ϵ), anti-CD3 ζ (ζ), and anti-CD3 γ (γ), and as reference (C) anti-HLA class I is shown. (Only β_2 M is shown here, 12 kDa.) The positions of the different chains are indicated on the right.

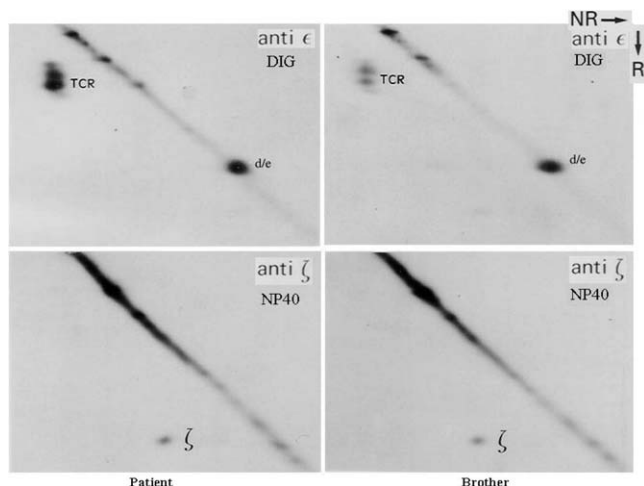


FIGURE 4 2-dimensional nonreducing/reducing SDS-PAGE analysis of anti-CD3 ϵ and anti- ζ immunoprecipitates. The T cells of the patient (left panel) and the healthy brother (right panel) were metabolically ^{35}S labeled, and lysates were prepared with digitonin (upper panel) or NP40 lysis buffer (lower panel). Antisera used were anti-CD3 ϵ (upper panel), anti-CD3 ζ (lower panel).

to 2-dimensional nonreducing/reducing SDS-PAGE analysis, which revealed normal intracellular expression of the disulfide-linked TCR $\alpha\beta$ dimers in the T cells of the healthy brother and the patient (Figure 4). Because of less efficient lysis in digitonin buffer as compared with NP40 buffer, the CD3 ζ and CD3 γ chains are not visible in the immunoprecipitates of the digitonin lysates (upper panel). The ζ chain, however, is visible in the anti- ζ precipitate of the NP40 lysates (lower panel). Thus, the patient synthesizes all TCR-CD3 chains except CD3 γ .

Influence of Amino-Acid Substitutions in CD3 γ on Assembly and Cell-Surface Expression of the TCR-CD3 Complex

Several amino acids in the binding interface between CD3 γ and CD3 ϵ have been implicated [20] in the specific interaction between these two chains (Figure 5, adapted from [21]). In particular, the amino acids at positions 76, 78, 82, and 85 in CD3 γ are thought to interact with amino acids in CD3 ϵ . Mutational analysis of CD3 ϵ has demonstrated that multiple replacements in the binding interface are required for abolishment of the CD3 $\gamma\epsilon$ interactions. Such an analysis has not been performed for CD3 γ . We have now taken advantage of the availability of the T-cell line of the CD3 γ -deficient patient to investigate this in detail. For this purpose, site-directed mutants of CD3 γ cDNA were generated that encode CD3 γ chains in which the amino acids thought to be important for the interaction with CD3 ϵ are substituted by either homologous or nonhomologous amino acids. These CD3 constructs, as well as wild type

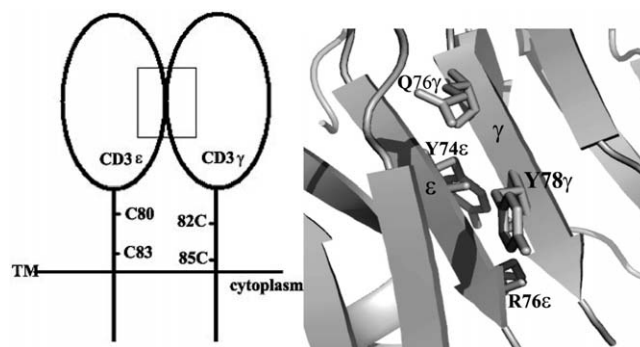


FIGURE 5 Schematic overview of the CD3 $\epsilon\gamma$ binding interface. The important residues are represented by their one-letter amino-acid code and their corresponding residue number. The right image, which is a close-up of the left picture, is adapted from [21] and prepared with PyMOL [44].

CD3 γ , were stably introduced into the patient's T-cell line together with the reporter gene, GFP, as described [26, 29], and GFP $^{+}$ cells were selected by FACS. As a control, the cells were transduced with an empty vector. FACS analysis demonstrated that the transduced cells were CD4 $^{+}\alpha\beta$ -TCR $^{+}$ (Figure 6A, B) and that the trans-

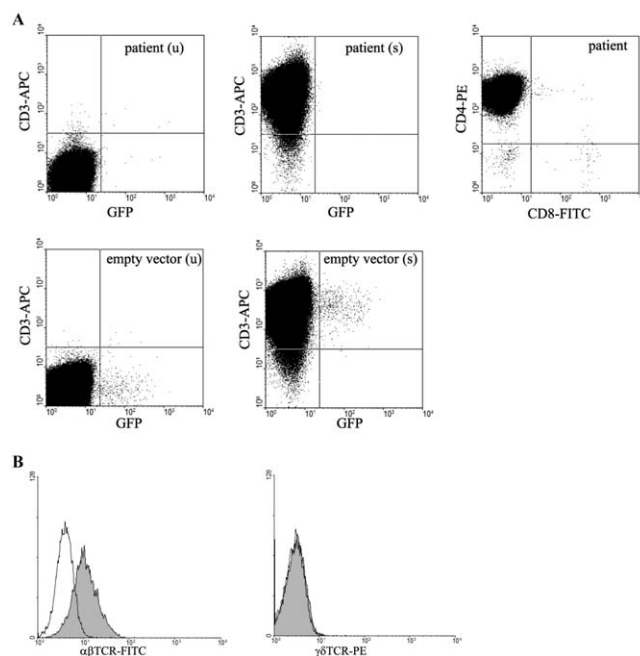


FIGURE 6 (A) FACS analysis of the T cells of the patient and the patient's T cells transduced with empty vector. The cells were unstained (u) or stained (s) with anti-CD3-PE or double-stained with CD4/CD8. (B) Histograms of patient's T cells. Cells were stained for the $\alpha\beta$ or $\gamma\delta$ T-cell receptor, respectively. Unstained cells are represented by a white histogram, and cells after staining are represented by a gray histogram. The abscissa gives the fluorescence intensity in a logarithmic scale; the ordinate gives the relative cell number.

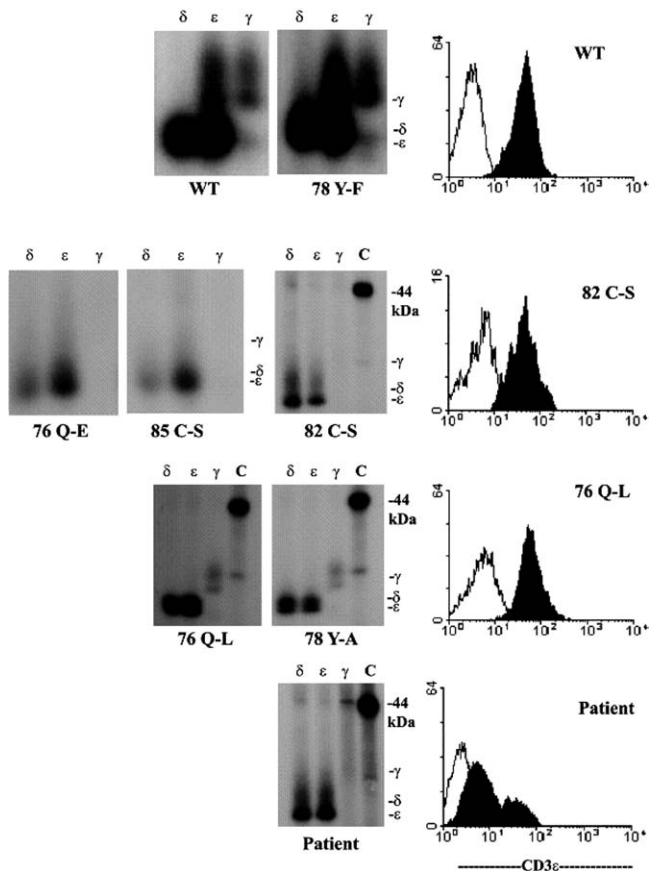


FIGURE 7 (Left) SDS-PAGE analysis of immunoprecipitates obtained from NP40 lysates of cell-surface iodinated cells from the patient and CD3 γ mutant cell lines. Antisera used were anti-CD3 δ (δ), anti-CD3 ϵ (ϵ), and anti-CD3 γ (γ), and anti-HLA class I (C, only heavy chain is shown). (Right) Histograms of patient and mutant GFP⁺ T-cells. Unstained cells are represented by a white histogram, and cells after staining with anti-CD3-PE are represented by a black histogram. The abscissa gives the fluorescence intensity in a logarithmic scale; the ordinate gives the relative cell number.

duction with the empty vector did not influence the CD3 expression on the patient's cells (Figure 6A).

The expression of the TCR-CD3 complex on the established GFP⁺ T-cell lines and the nontransfected T-cell line from the patient was determined by FACS analysis. Moreover, the intracellular and cell-surface expression of the CD3 γ , CD3 δ , and CD3 ϵ chains and the interaction between these chains were determined by SDS-PAGE analysis of CD3 δ , CD3 ϵ , and CD3 γ immunoprecipitates carried out with ³⁵S-labeled and ¹²⁵I-labeled cell lysates of the cell lines.

Normal expression of an intact TCR-CD3 $\delta\epsilon\gamma\zeta_2$ complex is found after the introduction of wild type CD3 γ and by the introduction of CD3 γ in which the tyrosine at position 78 is replaced by a phenylalanine (78Y-F; Figure 7, top panel). We therefore conclude that

the 78Y-F mutation allows the expression of a TCR-CD3 $\delta\epsilon\gamma\zeta_2$ complex on the cell surface. In contrast, CD3 γ was found not to be cell-surface expressed in the case of the CD3 γ mutants 82C-S, 85C-S, and 76Q-E (Figure 7, upper middle panel). To determine whether the mutated CD3 γ chain was synthesized, immunoprecipitation with the CD3 γ -specific antibody was carried out, using ³⁵S-labeled lysates of the transfectants. The results, shown for the CD3 γ mutant 85C-S, indicate that CD3 γ is synthesized at low levels (Figure 8). The intracellularly expressed CD3 γ , however, cannot associate with CD3 ϵ , because the $\alpha\beta$ TCR and CD3 ϵ could not be detected in the CD3 γ -specific immunoprecipitates carried out with digitonin (DIG) lysates. These cells thus appear to express TCR-CD3 $\delta\epsilon\delta\epsilon\zeta_2$.

Finally, in the case of the CD3 γ mutants 76Q-L and 78Y-A, the CD3 γ protein was cell-surface expressed despite an impaired association with CD3 ϵ (Figure 7, lower middle panel; Fig. 8). The presence of CD3 γ on the cell surface therefore indicates that these cells express TCR-CD3 $\delta\epsilon\gamma\zeta_2$ complexes.

DISCUSSION

The interaction of the TCR with its MHC-peptide ligand is a crucial step towards the initiation of adaptive immune responses. Because of the short cytoplasmic tails of the TCR α and β chains, however, these cannot transduce the signal over the cell membrane. This is accomplished by a still poorly understood mechanism through the TCR-associated CD3 γ , CD3 δ , CD3 ϵ , and CD3 ζ chains. Although several studies have indicated that the minimal TCR-CD3 complex contains 8 chains, $\alpha\beta$ TCR-CD3 $\delta\epsilon\gamma\zeta_2$, the exact stoichiometry of the complex is still unclear, as is the way in which all of the individual components are arranged in the complex. An NMR study has, for the first time, provided information on the structure of part of the extracellular domains of a

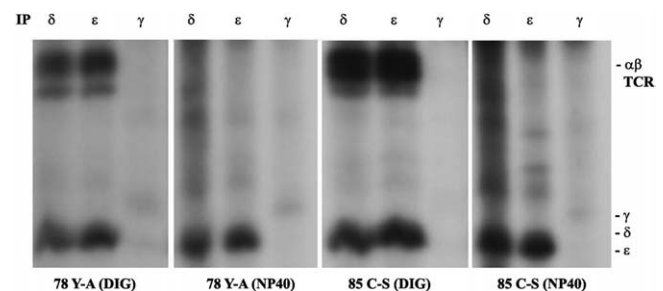


FIGURE 8 SDS-PAGE analysis of immunoprecipitates obtained from NP40 and digitonin (DIG) lysates after ³⁵S metabolic labeling of CD3 γ mutant cells. Antisera used were anti-CD3 δ (δ), anti-CD3 ϵ (ϵ), and anti-CD3 γ (γ). The positions of the different chains are indicated on the right.

murine CD3 γ ϵ complex [20]. In this study, several amino acids located in the interface between CD3 ϵ and CD3 γ were implicated as being important for the association. Mutational analysis of residues in the stalk region of CD3 ϵ indicated that combinations of mutations were required for disturbing the interaction of CD3 ϵ with CD3 γ . We have now generated mutants of CD3 γ and stably introduced these in T cells from a patient with a deleterious mutation in the CD3 γ gene, resulting in aberrant TCR-CD3 expression. TCR-CD3 complexes in such a patient are most likely composed of TCR-CD3 $\delta\epsilon\zeta$ complexes instead of TCR-CD3 $\delta\epsilon\gamma\zeta$ as has been suggested previously by Geisler [37] and Pérez-Aciego *et al.* [10]. In agreement with previous studies, we find that the reconstitution with wild type (wt) CD3 γ completely restores the cell-surface expression of an intact TCR-CD3 complex containing the TCR, CD3 δ , CD3 ϵ , and CD3 γ [9, 38]. In contrast, replacement of either of the two cysteine residues in the proximal stalk region of CD3 γ with serine abrogates the interaction between CD3 ϵ and CD3 γ . This is in disagreement with Sun *et al.* [20], who suggested that these conserved cysteines in the stalk region facilitate the CD3 ϵ γ pairing but are not required for the association of these CD3 chains. Our experiments, however, confirm the results discussed by Borroto *et al.* [39], suggesting the important participation of the stalk region of CD3 γ (containing the two cysteines 82C and 85C in a Cys-X-X-Cys motif) in pair formation with CD3 ϵ through the intrachain forming of a disulfide bridge.

The CD3 γ (78Y-F) mutation did not affect the CD3 γ ϵ association and thus led to the cell-surface expression of the TCR-CD3 $\delta\epsilon\gamma\zeta$ complex, comparable with that observed with wt CD3 γ . A striking result is that three mutations, 82C-S, 76Q-E, and 85C-S, did not result in a detectable incorporation of CD3 γ in the cell-surface-expressed TCR-CD3 complex as evidenced by a lack of (co)immunoprecipitation with the CD3 ϵ - and the CD3 γ -specific antibody. Intracellularly, however, the expression of these mutated CD3 γ chains could be detected as shown for mutant 85 C-S.

Finally, the 76Q-L and 78Y-A mutations led to intracellular expression and transport of CD3 γ to the cell surface, presumably in a TCR-CD3 $\delta\epsilon\gamma\zeta$ complex, in spite of an undetectable association with CD3 ϵ . This result indicates that a tight association between CD3 γ and CD3 ϵ is not required for normal expression of an intact TCR-CD3 complex.

It is important to note, however, that because of the culture conditions, the cells used for the transduction experiments already expressed relatively high levels of TCR-CD3 complexes, despite the absence of CD3 γ (Figure 6). Therefore, we can draw no conclusions regarding the impact of the mutations on the levels of cell-surface-

expressed TCR-CD3 complexes, but we can draw conclusions on the composition of these complexes. Irrespective of this, our results demonstrate the importance of several amino acids, including the two cysteine residues in the proximal stalk region, for the association between CD3 γ and CD3 ϵ .

Taking into consideration that a relatively large surface area of CD3 γ and CD3 ϵ appear to interact, it may seem surprising that single amino-acid substitutions can have such dramatic effects on the CD3 chain interactions. On the other hand, critical interactions between particular amino acids are likely to play a major role in stabilizing these CD3 chain interactions. In this respect, it is also important to note that studies in mice and humans have yielded conflicting results as to how many amino acids need to be altered to disturb the CD3 γ ϵ interaction.

Our findings are in agreement with, for example, Kappes and Tonegawa, [40] who suggested that CD3 ϵ and ζ chains are essential for reconstitution of the surface expression. This is in contrast to CD3 δ and CD3 γ , which can be used alternatively, thereby forming different types of TCR-CD3 complexes. Haks *et al.* [41], however, showed that CD3 γ is essential for development of the pre-TCR and could not be replaced by other CD3 components, whereas Dietrich *et al.* [42, 43] demonstrated that the extracellular domain of CD3 δ cannot substitute for the extracellular domain of CD3 γ in the assembly of the complete TCR in T cells. Clearly, therefore, contrasting results have been obtained in these various studies, which might relate to the use of different cell types in these studies.

In conclusion, our results have identified amino acids in the extracellular domain of CD3 γ that play a key role in the association between CD3 γ and CD3 ϵ , which is a prerequisite for the cell-surface expression of TCR-CD3 $\delta\epsilon\gamma\zeta$ complexes.

ACKNOWLEDGMENTS

The authors are indebted to Y.M. Kooy-Winkelaar for help with cell culturing and FACS analysis, to P.J. van den Elsen for providing the CD3 γ cDNA, and to Dr. G. Noles for the retroviral vectors. We thank Drs B.O. Roep and P.J. van den Elsen for critically reading the manuscript.

REFERENCES

1. Manolios N, Letourneur F, Bonifacio JS, Klausner RD: Pairwise, cooperative and inhibitory interactions describe the assembly and probable structure of the T-cell antigen receptor. *EMBO J* 10:1643, 1991.
2. San Jose E, Sahuquillo AG, Bragado R, Alarcon B: Assembly of the TCR/CD3 complex: CD3 epsilon/delta and CD3 epsilon/gamma dimers associate indistinctly with

- both TCR alpha and TCR beta chains. Evidence for a double TCR heterodimer model. *Eur J Immunol* 28:12, 1998.
3. Exley M, Wileman T, Mueller B, Terhorst C: Evidence for multivalent structure of T-cell antigen receptor complex. *Mol Immunol* 32:829, 1995.
 4. Fernandez-Miguel G, Alarcon B, Iglesias A, Bluethmann H, Alvarez-Mon M, Sanz E, de la Hera A: Multivalent structure of an alphabetaT cell receptor. *Proc Natl Acad Sci U.S.A* 96:1547, 1999.
 5. Wilson IA, Garcia KC: T-cell receptor structure and TCR complexes. *Curr Opin Struct Biol* 7:839, 1997.
 6. Davis MM, Boniface JJ, Reich Z, Lyons D, Hampl J, Arden B, Chien Y: Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol* 16:523–544, 1998.
 7. Garcia KC, Teyton L, Wilson IA: Structural basis of T cell recognition. *Annu Rev Immunol* 17:369, 1999.
 8. Germain RN: The T cell receptor for antigen: signaling and ligand discrimination. *J Biol Chem* 276:35223, 2001.
 9. Torres PS, Alcover A, Zapata DA, Arnaud J, Pacheco A, Martin-Fernandez JM, Villasevil EM, Sanal O, Regueiro JR: TCR dynamics in human mature T lymphocytes lacking CD3 gamma. *J Immunol* 170:5947, 2003.
 10. Perez-Aciego P, Alarcon B, Arnaiz-Villena A, Terhorst C, Timon M, Segurado OG, Regueiro JR: Expression and function of a variant T cell receptor complex lacking CD3-gamma. *J Exp Med* 174:319, 1991.
 11. Sommers CL, Dejarnette JB, Huang K, Lee J, El Khoury D, Shores EW, Love PE: Function of CD3 epsilon-mediated signals in T cell development. *J Exp Med* 192:913, 2000.
 12. Regueiro JR, Arnaiz-Villena A, Ortiz dL, Martin Villa JM, Vicario JL, Pascual-Ruiz V, Guerra-Garcia F, Alcamí J, Lopez-Botet M, Manzanares J: Familial defect of CD3 (T3) expression by T cells associated with rare gut epithelial cell autoantibodies. *Lancet* 1:1274, 1986.
 13. Arnaiz-Villena A, Timon M, Corell A, Perez-Aciego P, Martin-Villa JM, Regueiro JR: Brief report: primary immunodeficiency caused by mutations in the gene encoding the CD3-gamma subunit of the T-lymphocyte receptor. *N Engl J Med* 327:529, 1992.
 14. Soudais C, de Villartay JP, Le Deist F, Fischer A, Lisowska-Groszpiere B: Independent mutations of the human CD3-epsilon gene resulting in a T cell receptor/CD3 complex immunodeficiency. *Nat Genet* 3:77, 1993.
 15. Soudais C, de Villartay JP, Le Deist F, Fischer A, Lisowska-Groszpiere B: Genetic analysis of the human CD3-epsilon gene in a T cell receptor/CD3 immunodeficiency. *Immunodeficiency* 4:117, 1993.
 16. Thoenes G, Le Deist F, Fischer A, Griscelli C, Lisowska-Groszpiere B: Immunodeficiency associated with defective expression of the T-cell receptor-CD3 complex. *N Engl J Med* 322:1399, 1990.
 17. De Saint Basille G, Geissmann F, Flori E, Uring-Lambert B, Soudais C, Durandy M, Jabado N, Fischer A, Le Deist F. *J Clin Invest* 114(10):1512, 2004.
 18. Dadi HK, Simon AJ, Roifman CM: Effect of CD3delta deficiency on maturation of alpha/beta and gamma/delta T-cell lineages in severe combined immunodeficiency. *N Engl J Med* 349:1821, 2003.
 19. Zapata DA, Pacheco-Castro A, Torres PS, Millan R, Regueiro JR: CD3 immunodeficiencies. In Roifman CM (ed): *Immunology and allergy clinics of North America: inherited T cell immunodeficiencies*. Philadelphia, WB Saunders Co, 2000.
 20. Sun ZJ, Kim KS, Wagner G, Reinherz EL: Mechanisms contributing to T cell receptor signaling and assembly revealed by the solution structure of an ectodomain fragment of the CD3 epsilon gamma heterodimer. *Cell* 105: 913, 2001.
 21. Kjer-Nielsen L, Dunstone MA, Kostenko L, Ely LK, Beddoe T, Mifsud NA, Purcell AW, Brooks AG, McCluskey J, Rossjohn J: Crystal structure of the human T cell receptor CD3{ ϵ }{ γ } heterodimer complexed to the therapeutic mAb OKT3. *Proc Natl Acad Sci U.S.A* 101:7675, 2004.
 22. Sun ZY, Kim ST, Kim IC, Fahma A, Reinherz EL and Wagner G: Solution structure of the CD3 $\epsilon\delta$ ectodomain and comparison with CD3 $\epsilon\gamma$ as a basis for modeling T cell receptor topology and signaling. *Proc Natl Acad Sci U.S.A* 101:16867, 2004.
 23. Arnett KL, Harrison SC, Wiley DC: Crystal structure of a human CD3-epsilon/delta dimer in complex with a UCHT1 single-chain antibody fragment. *Proc Natl Acad Sci U.S.A* 101:16268, 2004.
 24. Call ME, Pyrdol J, Wucherpfennig KW: Stoichiometry of the T-cell-receptor-CD3 complex and key intermediates assembled in the endoplasmic reticulum. *EMBO J* 23:2348, 2004.
 25. Call ME, Wucherpfennig KW: Molecular mechanisms for the assembly of the T cell receptor-CD3 complex. *Mol Immunol* 40:1295, 2004.
 26. Heemskerk MH, Blom B, Nolan G, Stegmann AP, Bakker AQ, Weijer K, Res PC, Spits H: Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J Exp Med* 186:1597, 1997.
 27. Landt O, Grunert HP, Hahn U: A general method for rapid site-directed mutagenesis using the polymerase chain reaction. *Gene* 96:125, 1990.
 28. Krissansen GW, Owen MJ, Verbi W, Crumpton MJ: Primary structure of the T3 gamma subunit of the T3/T cell antigen receptor complex deduced from cDNA sequences: evolution of the T3 gamma and delta subunits. *EMBO J* 5:1799, 1986.
 29. Kinsella TM, Nolan GP: Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum Gene Ther* 7:1405, 1996.

30. Koning F, Maloy WL, Coligan JE: The implications of subunit interactions for the structure of the T cell receptor-CD3 complex. *Eur J Immunol* 20:299, 1990.
31. Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, Ziegler A: Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. *Cell* 14:9, 1978.
32. Borst J, van Dongen JJ, de Vries E, Comans-Bitter WM, van Tol Mj, Vossen JM, Kurrle R: BMA031, a monoclonal antibody suited to identify the T-cell receptor alpha beta/CD3 complex on viable human T lymphocytes in normal and disease states. *Hum Immunol* 29:175, 1990.
33. Sanal O, Yel L, Ersoy F, Tezcan I, Berkel AI: Low expression of T-cell receptor-CD3 complex: a case with a clinical presentation resembling humoral immunodeficiency. *Turk J Pediatr* 38:81, 1996.
34. Zapata DA, Pacheco-Castro A, Torres PS, Ramiro AR, Jose ES, Alarcon B, Alibaud L, Rubin B, Toribio ML, Regueiro JR: Conformational and biochemical differences in the TCR CD3 complex of CD8(+) versus CD4(+) mature lymphocytes revealed in the absence of CD3gamma. *J Biol Chem* 274:35119, 1999.
35. Torres PS, Zapata DA, Pacheco-Castro A, Rodriguez-Fernandez JL, Cabanas C, Regueiro JR: Contribution of CD3 gamma to TCR regulation and signaling in human mature T lymphocytes. *Int Immunol* 14:1357, 2002.
36. Van Neerven J, Coligan JE, Koning F: Structural comparison of alpha/beta and gamma/delta T cell receptor-CD3 complexes reveals identical subunit interactions but distinct cross-linking patterns of T cell receptor chains. *Eur J Immunol* 20:2105, 1990.
37. Geisler C: Failure to synthesize the CD3-gamma chain. Consequences for T cell antigen receptor assembly, processing, and expression. *J Immunol* 148:2437, 1992.
38. Sun JY, Pacheco-Castro A, Borroto A, Alarcon B, Alvarez-Zapata D, Regueiro JR: Construction of retroviral vectors carrying human CD3 gamma cDNA and reconstitution of CD3 gamma expression and T cell receptor surface expression and function in a CD3 gamma-deficient mutant T cell line. *Hum Gene Ther* 8:1041, 1997.
39. Borroto A, Mallabiabarrena A, Albar JP, Martinez A, Alarcon B: Characterization of the region involved in CD3 pairwise interactions within the T cell receptor complex. *J Biol Chem* 273:12807, 1998.
40. Kappes DJ, Tonegawa S: Surface expression of alternative forms of the TCR/CD3 complex. *Proc Natl Acad Sci U.S.A* 88:10619, 1991.
41. Haks MC, Krimpenfort P, Borst J, Kruisbeek AM: The CD3gamma chain is essential for development of both the TCR alphabeta and TCR gammadelta lineages. *EMBO J* 17:1871, 1998.
42. Dietrich J, Neisig A, Hou X, Wegener AM, Gajhede M, Geisler C: Role of CD3 gamma in T cell receptor assembly. *J Cell Biol* 132:299, 1996.
43. Wegener AM, Hou X, Dietrich J, Geisler C: Distinct domains of the CD3-gamma chain are involved in surface expression and function of the T cell antigen receptor. *J Biol Chem* 270:4675, 1995.
44. DeLano WL: The PyMOL molecular graphics system. <http://www.pymol.org>.