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The Impact of Single Amino Acid Substitutions in CD3γ on the CD3εγ Interaction and T-Cell Receptor–CD3 Complex Formation

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ABSTRACT: The human T-cell receptor–CD3 complex consists of at least eight polypeptide chains; CD3γε- and δε-dimers associate with the disulfide linked αβ- and ζζ-dimers to form a functional receptor complex. The exact structure of this complex is still unknown. We now have examined the interaction between CD3γ and CD3ε in human T-cells. For this purpose, we have generated site-directed mutants of CD3γ that were introduced in human T-cells defective in CD3γ expression. Cell-surface and intracellular expression of the introduced CD3γ chains was determined, as was the association with CD3δ, CD3ε, and the T-cell receptor. Although the introduction of wild type CD3γ and CD3γ (78Y-F) fully restored T-cell receptor assembly and expression, the introduction of CD3γ (82C-S), CD3γ (85C-S), and CD3γ (76Q-E) all resulted in an impaired association between CD3γ and CD3ε and a lack of cell-surface expressed CD3γ. Finally, the introduction of CD3γ (76Q-L) and CD3γ (78Y-A) restored the expression of TCR-CD3δεγεζ_2 complexes, although the association between CD3γ and CD3ε was impaired. These results indicate that several amino acids in CD3γ are essential for an optimal association between CD3γ and CD3ε and the assembly of a cell-surface expressed TCR-CD3δεγεζ_2 complex. *Human Immunology 67, 579–588 (2006).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: CD3γ 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 histocompatiblity 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 αβ T-cell receptor (TCR) heterodimer. For the full function of the receptor, the disulfide-linked αβ-chains associate with CD3δ, CD3ε, CD3γ, and ζ. These latter form noncovalently linked δε and εγ heterodimers and disulfide-linked ζ-ζ homodimers. While each TCR-CD3

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complex therefore consists of a minimum of eight polypeptides, the exact stoichiometry of the complex is still under discussion [1–4]. The TCRαβ, 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]. CD3δ, CD3ε, and CD3γ 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 CD3γ 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 CD3ε 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 CD3ε deficiency. In 2003, Dadi *et al.* [18] studied three cases of CD3δ deficiency. In total, three cases of human CD3γ 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 CD3γ 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 CD3εγ heterodimer. Mutational analysis of CD3ε, focusing on the binding interface between CD3ε and CD3γ, implicated several amino acids in CD3ε and CD3γ as being important for the domain–domain interaction. In this analysis, combinations of mutations in CD3ε were found to be required for strong effects on the association between CD3ε and CD3γ. More recently, the structure of the human CD3εγ 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 origins of the heterodimers (human CD3ε and CD3γ 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 CD3εδ ectodomain [22] and the crystal structure of the CD3εδ 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 αβTCR-CD3 complex is composed of one copy of the αβTCR, CD3δε, CD3γε and ζζ dimers. The assembly of this TCR-CD3 complex was found to follow discrete steps in which the transmembrane region of CD3δε, CD3γε and ζζ 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 αβTCR and serve as critical points for assembly with CD3δε and CD3γε dimers, whereas the ζζ dimer associates with the arginine residue present in the αTCR.

In the present study we have further analyzed the binding interface of the CD3εγ dimer in human T-cells by introducing mutations in CD3γ and determining the ability of such mutated CD3γ chains to form CD3γε dimers and participate in the formation of cell-surface–expressed TCR-CD3 complexes. The results demonstrate that single amino acid alterations in CD3γ can have a significant effect on the composition of the cell-surface–expressed TCR-CD3 complex and provide further evidence that αβTCRδεδεζζ complexes can be formed even when the association of CD3εγ-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 μg/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 μg/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, LZRSpBMN-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 CD3γ 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 μg/mL puromycin (Clontech, Palo Alto, CA, USA) were added. At 10 to 14 days after transfection, 6×10^6 cells per 10-cm^2 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 retronectin (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 μg/mL phytohemagglutinin, and 10×10^6/mL irradiated (2500 rad) PMBCs as feeder cells.

Antibodies

The antibodies against CD3δ, CD3ε, and CD3γ were all rabbit antibodies and directed against peptides corresponding to the carboxy termini of the human CD3 chains. The anti-CD3ζ 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 αβTCR-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 μl lactoperoxidase (250 U/mL; Sigma, Zwijndrecht, The Netherlands) solution. One mCi $Na^{125}I$ (NEN) was added to the cells followed by the addition of 10 μl 0.05% H_2O_2/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 μl 2 mM KI/PBS, the cells were solubilized in 750 μl 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 × g) at 4°C. For ^{35}S 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% CO_2 and centrifuged, and the resulting pellets were resuspended in 10 mL RPMI without methionine/cysteine, 100 U/mL IL2, and 1 mCi ^{35}S-methionine/cysteine. After 4 hours of incubation at 37°C /5% CO_2, cells were centrifuged and washed twice with PBS. The cells were

solubilized in 750 μl 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 × g) at 4°C.

Immunoprecipitation and SDS-PAGE Analysis

The lysates, after labeling with ^{125}I or ^{35}S, were precleared by adding 100 μL protein A sepharose CL4B (PAS beads, Amersham Pharmacia Biotech, Roosendaal, The Netherlands), gently shaken at room temperature, followed by the addition of 75 μL normal rabbit serum and shaken at room temperature for 1 hour. After the removal of the beads, specific immunoprecipitations with CD3δ, CD3ϵ, CD3ζ, or CD3γ antibodies, normal rabbit serum, or anti-HLA class I were performed by adding 5 μL antiserum (2 μL for anti-HLA class I) to 100 μL precleared lysate (1D SDS-PAGE) or 200 μL precleared lysate (2D SDS-PAGE) for 60 minutes at room temperature. The PAS beads were washed four times with 500 μL lysis buffer and resuspended in 100 μL 1× 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/αβ-TCR double stainings, cells were first incubated with anti-αβ-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.

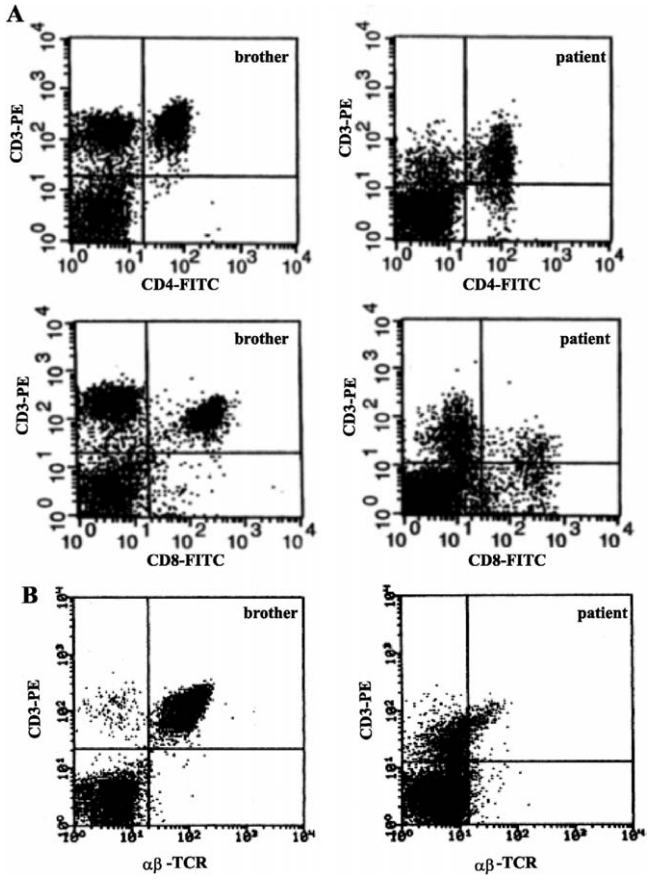

FIGURE 1 (A) FACS analysis of double-stained PBMCs of the patient (right panel) and healthy brother (left panel). The upper panel shows the CD3/CD4 stainings, and the lower panel shows the CD3/CD8 staining. (B) FACS analysis of T-cell lines established from PBMCs of the patient and brother. The left panel shows the CD3/αβ-TCR staining of the brother, and the right panel shows the CD3/αβ-TCR analysis for the CD3γ-deficient patient. Whereas 96% of the T cells of the brother were $\alpha\beta TCR^+$, only2% of the patient's cells were $\alpha\beta TCR^+$.

RESULTS

Characterization of TCR-CD3 Expression on T Cells from a Patient with a CD3γ Deficiency

A male patient was diagnosed with CD3γ deficiency at the age of 4 [33]. His parents are first cousins. An AAA-to-TAA mutation at position 242 of CD3γ 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.

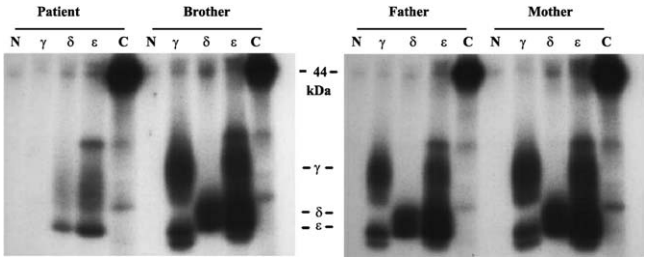

FIGURE 2 SDS-PAGE analyses of immunoprecipitates obtained from NP40 lysates after cell-surface iodination of T-cell lines of patient, brother, father, and mother. Antisera used were normal rabbit serum as negative control (N), anti-CD3δ (δ), anti-CD3ϵ (ϵ), and anti-CD3γ (γ), and as reference (C) anti-HLA 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 CD3γ-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 CD3γ-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 αβTCR 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 CD3γ-, δ-, and ϵ-chains, the cell lines were labeled with ^{125}I and lysed in NP40 lysis buffer. Subsequently, immunoprecipitations were carried out with CD3δ-, CD3ϵ-, and CD3γ-specific antibodies, followed by 1-dimensional SDS-PAGE analysis (Figure 2). In NP40 lysis buffer the TCR-CD3 complex dissociates into TCRαβ, ζ, CD3γϵ, and CD3δϵ dimers [36]. Consequently, CD3γ and CD3ϵ are present in CD3γ immunoprecipitates, CD3δ and CD3ϵ in CD3δ immunoprecipitates, and all three CD3 chains in CD3ϵ immunoprecipitates. Although in the lysates of the cell lines from the patient's healthy brother and his parents CD3δ, CD3ϵ, and CD3γ were present and associated as expected, the lysate of the cell line of the CD3γ-deficient patient contained very little CD3δ and CD3ϵ, and CD3γ was undetectable (Figure 2). Also, the mobility of CD3δ 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 CD3δ as described [34, 35].

To verify the presence of TCR-CD3 components intracellularly, the T-cell lines were labeled with ^{35}S-methionine/cysteine, and after lysis in NP40 lysis buffer, specific immunoprecipitations were carried out with CD3γ-, CD3δ, CD3ϵ-, and CD3ζ-specific antibodies, followed by SDS-PAGE analysis. This demonstrated (Figure 3) that the CD3γ, CD3δ, CD3ϵ, and CD3ζ 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 CD3δ, CD3ϵ, and CD3ζ chains at levels comparable with the healthy controls but no CD3γ. Both parents are heterozygous for the CD3γ mutation, which may explain the apparently diminished level of CD3γ as compared with their healthy son (Figure 3). The presence of CD3γ in both parents, however, is proven by the presence of CD3ϵ in the immunoprecipitates of NP40 lysis performed with an antibody against CD3γ. 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 TCRαβ chains, immunoprecipitates were obtained with the anti-CD3ϵ 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

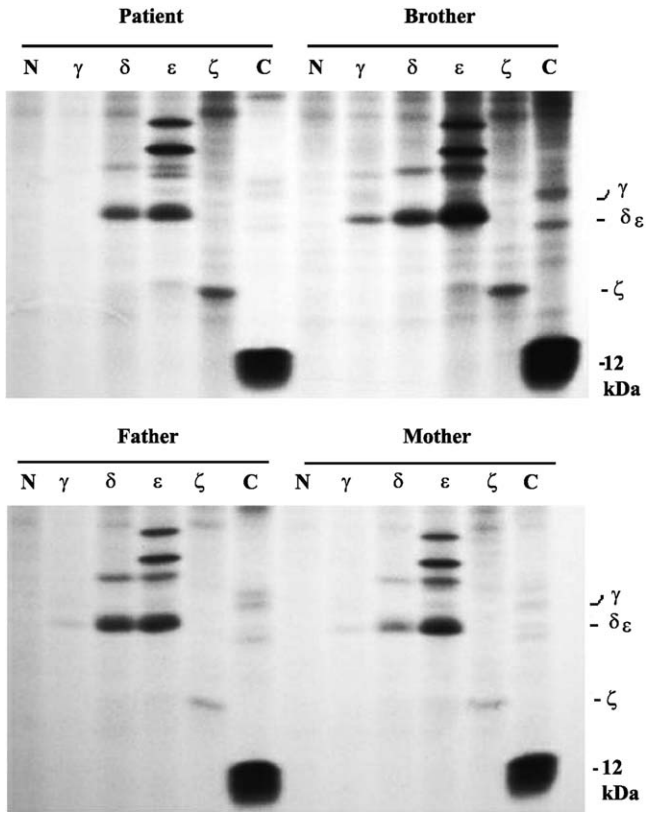

FIGURE 3 SDS-PAGE analysis of immunoprecipitates obtained from NP40 lysates after metabolic labeling of T-cell lines of patient, brother, father, and mother. Antisera used were normal rabbit serum as negative control (N), anti-CD3δ (δ), anti-CD3ϵ (ϵ), anti-CD3ζ (ζ), and anti-CD3γ (γ), and as reference (C) anti-HLA class I is shown. (Only β_2M is shown here, 12 kDa.) The positions of the different chains are indicated on the right.

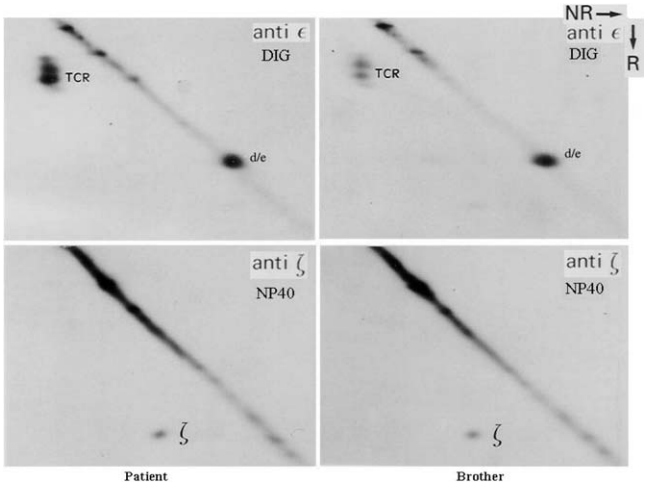

FIGURE 4 2-dimensional nonreducing/reducing SDS-PAGE analysis of anti-CD3ϵ and anti-ζ immunoprecipitates. The T cells of the patient (left panel) and the healthy brother (right panel) were metabolically ^{35}S labeled, and lysates were prepared with digitonin (upper panel) or NP40 lysis buffer (lower panel). Antisera used were anti-CD3ϵ (upper panel), anti-CD3ζ (lower panel).

to 2-dimensional nonreducing/reducing SDS-PAGE analysis, which revealed normal intracellular expression of the disulfide-linked TCRαβ 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 CD3ζ and CD3γ chains are not visible in the immunoprecipitates of the digitonin lysates (upper panel). The ζ chain, however, is visible in the anti-ζ precipiate of the NP40 lysates (lower panel). Thus, the patient synthesizes all TCR-CD3 chains except CD3γ.

Influence of Amino-Acid Substitutions in CD3γ on Assembly and Cell-Surface Expression of the TCR-CD3 Complex

Several amino acids in the binding interface between CD3γ and CD3ϵ 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 CD3γ are thought to interact with amino acids in CD3ϵ. Mutational analysis of CD3ϵ has demonstrated that multiple replacements in the binding interface are required for abolishment of the CD3γϵ interactions. Such an analysis has not been performed for CD3γ. We have now taken advantage of the availability of the T-cell line of the CD3γ-deficient patient to investigate this in detail. For this purpose, site-directed mutants of CD3γ cDNA were generated that encode CD3γ chains in which the amino acids thought to be important for the interaction with CD3ϵ are substituted by either homologous or nonhomologous amino acids. These CD3 constructs, as well as wild type

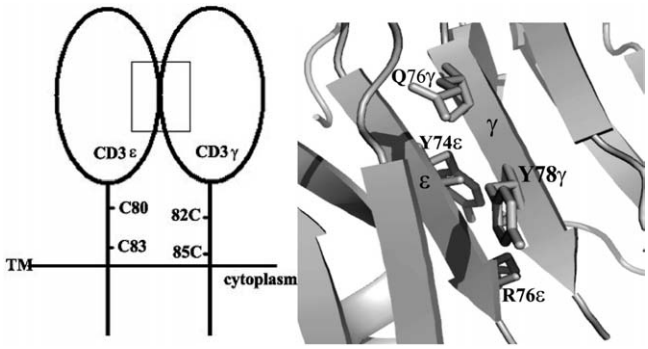

FIGURE 5 Schematic overview of the CD3ϵγ binding interface. The important residues are represented by their one-letter amino-acid code and their corresponding residue number. The right image, which is a close-up of the left picture, is adapted from [21] and prepared with PyMOL [44].

CD3γ, 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 tranduced cells were $CD4^+$αβ-TCR^+ (Figure 6A, B) and that the trans-

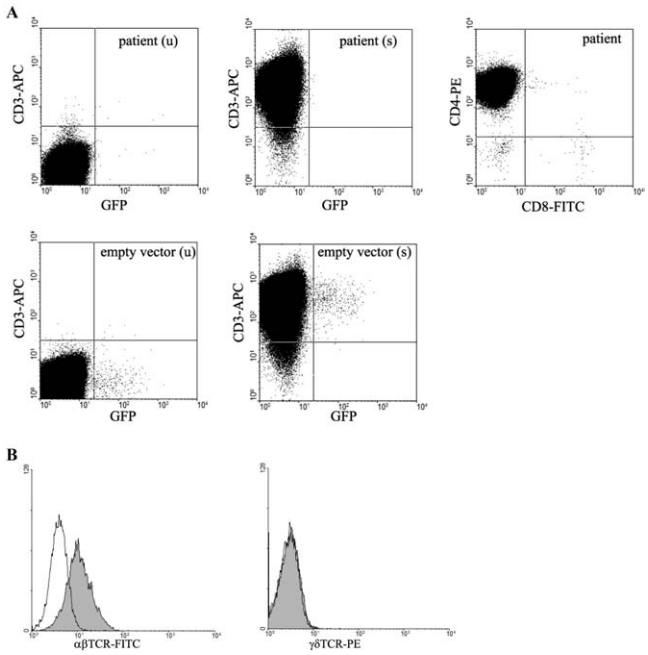

FIGURE 6 (A) FACS analysis of the T cells of the patient and the patient's T cells transduced with empty vector. The cells were unstained (u) or stained (s) with anti-CD3-PE or double-stained with CD4/CD8. (B) Histograms of patient's T cells. Cells were stained for the αβ or γδ T-cell receptor, respectively. 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.

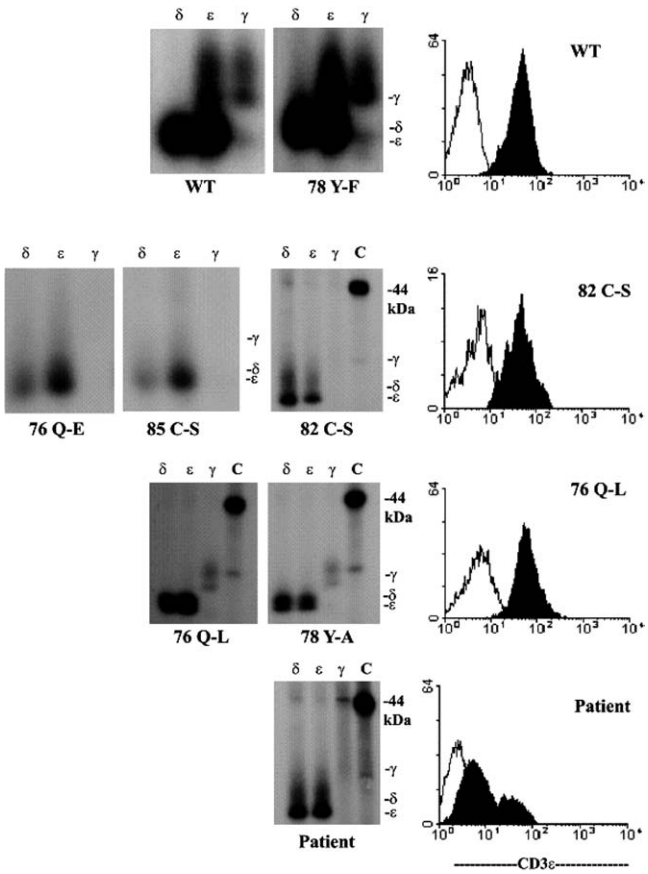

FIGURE 7 (Left) SDS-PAGE analysis of immunoprecipitates obtained from NP40 lysates of cell-surface iodinated cells from the patient and CD3γ mutant cell lines. Antisera used were anti-CD3δ (δ), anti-CD3ε (ε), and anti-CD3γ (γ), and anti-HLA class I (C, only heavy chain is shown). (Right) Histograms of 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 CD3γ, CD3δ, and CD3ε chains and the interaction between these chains were determined by SDS-PAGE analysis of CD3δ, CD3ε, and CD3γ immunoprecipitates carried out with ^{35}S-labeled and ^{125}I-labeled cell lysates of the cell lines.

Normal expression of an intact TCR-CD3δεγεζ_2 complex is found after the introduction of wild type CD3γ and by the introduction of CD3γ 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-CD3δεγεζ_2 complex on the cell surface. In contrast, CD3γ was found not to be cell-surface expressed in the case of the CD3γ mutants 82C-S, 85C-S, and 76Q-E (Figure 7, upper middle panel). To determine whether the mutated CD3γ chain was synthesized, immunoprecipitation with the CD3γ-specific antibody was carried out, using ^{35}S-labeled lysates of the transfectants. The results, shown for the CD3γ mutant 85C-S, indicate that CD3γ is synthesized at low levels (Figure 8). The intracellularly expressed CD3γ, however, cannot associate with CD3ε, because the αβTCR and CD3ε could not be detected in the CD3γ-specific immunoprecipitates carried out with digitonin (DIG) lysates. These cells thus appear to express TCR-CD3δεδεζ_2.

Finally, in the case of the CD3γ mutants 76Q-L and 78Y-A, the CD3γ protein was cell-surface expressed despite an impaired association with CD3ε (Figure 7, lower middle panel; Fig. 8). The presence of CD3γ on the cell surface therefore indicates that these cells express TCR-CD3δεγεζ_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 CD3γ, CD3δ, CD3ε, and CD3ζ chains. Although several studies have indicated that the minimal TCR-CD3 complex contains 8 chains, αβTCR-CD3δεγεζζ, 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

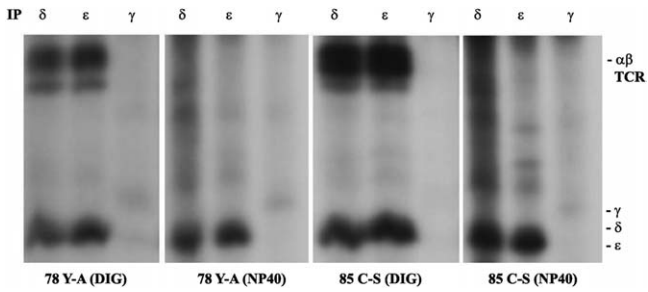

FIGURE 8 SDS-PAGE analysis of immunoprecipitates obtained from NP40 and digitonin (DIG) lysates after ^{35}S metabolic labeling of CD3γ mutant cells. Antisera used were anti-CD3δ (δ), anti-CD3ε (ε), and anti-CD3γ (γ). The positions of the different chains are indicated on the right.

murine CD3γε complex [20]. In this study, several amino acids located in the interface between CD3ε and CD3γ were implicated as being important for the association. Mutational analysis of residues in the stalk region of CD3ε indicated that combinations of mutations were required for disturbing the interaction of CD3ε with CD3γ. We have now generated mutants of CD3γ and stably introduced these in T cells from a patient with a deleterious mutation in the CD3γ gene, resulting in aberrant TCR-CD3 expression. TCR-CD3 complexes in such a patient are most likely composed of TCR-CD3δεδεζζ complexes instead of TCR-CD3δεγεζζ 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) CD3γ completely restores the cell-surface expression of an intact TCR-CD3 complex containing the TCR, CD3δ, CD3ε, and CD3γ [9, 38]. In contrast, replacement of either of the two cysteine residues in the proximal stalk region of CD3γ with serine abrogates the interaction between CD3ε and CD3γ. This is in disagreement with Sun *et al.* [20], who suggested that these conserved cysteines in the stalk region facilitate the CD3εγ 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 CD3γ (containing the two cysteines 82C and 85C in a Cys-X-X-Cys motif) in pair formation with CD3ε through the intrachain forming of a disulfide bridge.

The CD3γ (78Y-F) mutation did not affect the CD3γε association and thus led to the cell-surface expression of the TCR-CD3δεγεζζ complex, comparable with that observed with wt CD3γ. A striking result is that three mutations, 82C-S, 76Q-E, and 85C-S, did not result in a detectable incorporation of CD3γ in the cell-surface–expressed TCR-CD3 complex as evidenced by a lack of (co)immunoprecipitation with the CD3ε- and the CD3γ-specific antibody. Intracellularly, however, the expression of these mutated CD3γ 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 CD3γ to the cell surface, presumably in a TCR-CD3δεγεζζ complex, in spite of an undetectable association with CD3ε. This result indicates that a tight association between CD3γ and CD3ε 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 CD3γ (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 CD3γ and CD3ε.

Taking into consideration that a relatively large surface area of CD3γ and CD3ε 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 CD3γε interaction.

Our findings are in agreement with, for example, Kappes and Tonegawa, [40] who suggested that CD3ε and ζ chains are essential for reconstitution of the surface expression. This is in contrast to CD3δ and CD3γ, which can be used alternatively, thereby forming different types of TCR-CD3 complexes. Haks *et al.* [41], however, showed that CD3γ 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 CD3δ cannot substitute for the extracellular domain of CD3γ 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 CD3γ that play a key role in the association between CD3γ and CD3ε, which is a prerequisite for the cell-surface expression of TCR-CD3δεγεζζ complexes.

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