Role of CD3γ in T Cell Receptor Assembly

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Abstract. The T cell receptor (TCR) consists of the Tiαβ heterodimer and the associated CD3γδε and ζ_2 chains. The structural relationships between the subunits of the TCR complex are still not fully known. In this study we examined the role of the extracellular (EC), transmembrane (TM), and cytoplasmic (CY) domain of CD3γ in assembly and cell surface expression of the complete TCR in human T cells. A computer model indicated that the EC domain of CD3γ folds as an Ig domain. Based on this model and on alignment studies, two potential interaction sites were predicted in the EC domain of CD3γ. Site-directed mutagenesis demonstrated that these sites play a crucial role in TCR assembly probably by binding to CD3ε. Mutagenesis of N-linked glycosylation sites showed that glycosylation of CD3γ is not required for TCR assembly and expression. In contrast, treatment of T cells with tunicamycin suggested that N-linked glycosylation of CD3δ is required for TCR assembly. Site-directed mutagenesis of the acidic amino acid in the TM domain of CD3γ demonstrated that this residue is involved in TCR assembly probably by binding to Tiβ. Deletion of the entire CY domain of CD3γ did not prevent assembly and expression of the TCR. In conclusion, this study demonstrated that specific TCR interaction sites exist in both the EC and TM domain of CD3γ. Furthermore, the study indicated that, in contrast to CD3γ, glycosylation of CD3δ is required for TCR assembly and expression.

THE T cell receptor (TCR)[1] consists of the disulfide linked Tiαβ heterodimer and the non-covalently associated CD3γδε and ζ_2 chains. The function of the Tiαβ dimer is to recognize antigen presented on major histocompatibility complex encoded class I or class II molecules on the surface of antigen presenting cells (15, 49), whereas the major role of the CD3 and the ζ chains is to mediate activation signals and to regulate the level of TCR expression (16, 38, 57). All of the TCR chains are type I integral membrane proteins (12, 30, 50). Except for ζ the TCR chains belong to the immunoglobulin (Ig) superfamily (10, 22, 35, 60).

All the TCR chains are required for efficient TCR expression at the cell surface (8), but despite extensive research the subunit interactions within the TCR are still not fully understood. Several studies have described the presence of intracellular CD3δε, γε, ζζ, and Tiαβ dimers (2, 4, 43) and these dimers can form independently of other TCR chains. Transfection of TCR chains into COS cells has demonstrated a preferential binding of Tiα to CD3δε dimers (2) and cross-linking experiments have shown that Tiβ is in close contact to CD3γ and to a lesser extent to CD3ε at the surface of T cells (6, 31, 52). From these results a TCR model has been proposed in which CD3δε dimers associate with Tiα and CD3γε dimers associate with Tiβ (41). It is presently thought that ζ_2 associates with this putative hexameric partial TCR complex via a compound determinant created by the complex. The specific chains that interact with ζ_2 are not known although experiments have indicated that Tiα and β are involved (20, 21).

Recent experiments have indicated that the extracellular (EC) domains of the TCR chains are involved in assembly of the TCR. One study demonstrated that the association of CD3 chains with Tiβ was stabilized by the EC constant (C) domain of the Tiβ chain (59). In another experiment it was shown that the assembly of Tiα and Tiβ was dependent on the EC domains of these chains (41). Both experiments involved the use of COS cells. As different conditions for TCR assembly might exist in non-T cells compared with T cells, conclusions on TCR assembly based on results obtained with non-T cells should be drawn with caution. We have recently demonstrated that the EC domain of CD3δ can not substitute for the EC domain of CD3γ in assembly of the complete TCR in T cells (58). This indicates that the EC domain of CD3γ has a specific role in TCR assembly and expression.

The CD3γ chain contains two N-linked glycosylation sites in the EC domain. The general role of protein glycosylation is still not fully understood. However, experiments

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1. *Abbreviations used in this paper*: C, extracellular constant; CY, cytoplasmic; EC, extracellular; PA, protein A–Sepharose; PCR, polymerase chain reaction; PE, phycoerythrin; TCR, T cell receptor; TM, transmembrane.

have shown that glycosylation might be involved in stabilizing the structure of proteins, in modifying the functional activity of proteins, in recognition events, and in signal transduction after ligand-receptor binding (reviewed in reference 53). In addition, it has recently been demonstrated that mutation of a glycosylation site in the transferrin receptor has a profound negative effect on the appearance of the receptor on the cell surface (61). The role of CD3γ glycosylation in assembly, intracellular transport and expression of the TCR is unknown.

Each of the TCR chains contains one or two charged amino acids in their transmembrane (TM) domain. Several studies have demonstrated a requirement of these charged amino acids for complete TCR assembly. Mutation of both the TM basic amino acids in Tiα abolished TCR expression in T cells (3, 26). Furthermore, in COS cells the association of Tiα with CD3δ was impaired if both the TM basic amino acids in Tiα were changed to Leu or if the TM acidic amino acid in CD3δ was changed to Leu (42). Point mutation of the Tiβ TM basic amino acid also abolished TCR expression in T cells due to the inability of the mutated Tiβ chain to associate with the CD3 chains (1). In agreement with this, it has been shown in COS cells that if the charged amino acid in the TM domain of any of the CD3 chains was changed to a hydrophobic amino acid the association between CD3 and the Ti chains was abolished, whereas the CD3–CD3 associations were not dependent on the presence of these charged amino acids (23). These experiments indicate that the charged TM amino acids of the TCR subunits play an import role in the interaction between Ti and CD3 chains. However, the role of the charged amino acid in the TM domain of CD3γ in assembly of the complete TCR has not been studied in T cells. Furthermore, the role of the cytoplasmic tail of CD3γ in TCR assembly and surface expression is unknown.

On the basis of a computer model and alignment studies we identified sites in the EC domain of CD3γ that could be involved in interactions with other TCR chains. The role of these sites in TCR assembly was subsequently analyzed by transfecting site-directed mutagenized CD3γ cDNA into the CD3γ negative T cell variant JGN (18). Furthermore, the role of N-linked glycosylation of the EC domain, the role of the charged residue in the TM domain, and the role of the cytoplasmic (CY) domain of CD3γ for TCR assembly and expression was studied.

Materials and Methods

Computer Model Building

The model of CD3γ was built using software programs from Biosym Technologies (San Diego, CA). The initial model was built using Homology® and three structures (44, 48, 55) all containing the Ig superfamily fold. The resulting structure was energy minimized using the Discover® program and the CFF91 forcefield and molecular dynamics were subsequently performed. The final structure was analyzed using program PROCHECK (37), and the secondary structural elements were identified based on the Kabsh-Sander algorithm (27).

Cells and Medium

JGN cells, a TCR cell surface negative variant of the human T cell line Jurkat that synthesize no CD3γ (18), and the original Jurkat cell line, J76, were cultured in RPMI 1640 medium (GIBCO BRL, Paisley, UK) supplemented with penicillin, 0.5 IU/l (Leo, Ballerup, Denmark), streptomycin, 500 mg/l (Novo, Bagsvœrd, Denmark), and 10% (vol/vol) FCS (Sera-Lab Ltd., Sussex, UK) at 37°C in 5% CO_2. The Tiβ deficient Jurkat variant JBN (49) was kindly provided by Dr. R. N. Germain (Bethesda, MD).

Antibodies and Chemicals

The UCHT1 mouse mAb directed against human CD3ε was obtained purified and phycoerythrin (PE) conjugated from Dakopatts A/S (Glostrup, Denmark). F101.01 mouse mAb against a conformational epitope on CD3γε and CD3δε dimers in association with the Ti chains was produced in our own laboratory (19). HMT-3.2 hamster mAb against an epitope on the IC domain of human CD3γ and mouse CD3γ and δ was kindly donated by Dr. Ralph Kubo (Cytel Corporation, San Diego, CA).

Constructs and Generation of Stably Transfected Cells

All mutations and truncations were constructed as previously described (16, 18, 57) by the polymerase chain reaction (PCR) using Vent DNA polymerase containing 3′→5′ proofreading exonuclease activity (New England Biolabs, Inc., Beverly, MA) and the wild-type human CD3γ cDNA containing plasmid pJ6T3γ-2 (33) as template. PCR products were cut with XbaI-NcoI, cloned into the 4.1-kb XbaI-NcoI fragment of pBluescript-βWT (18) and confirmed by complete DNA sequencing. The 1.8-kb XbaI-BamHI pBluescript fragment containing the PCR product was subsequently cloned into the 5.9-kb XbaI-BamHI fragment of the expression vector pTβFneo (45). Transfections were performed using the Bio-Rad Gene Pulser at a setting of 270 V and 960 μF with 40 μg of plasmid per 2×10^7 cells. After electroporation, cells were maintained in RPMI medium for 24 h and plated at 1×10^4 and 5×10^4 cells/ml in 96-well tissue culture plates (Greiner GmbH, Frickenhausen, Germany) in medium containing 1 mg/ml G418 sulphate (Geneticin) (GIBCO BRL). After 3–4 wk of selection, G418 resistant clones were expanded and maintained in medium without G418.

Flow Cytometric Analysis

Cells were washed twice in ice cold PBS, 0.02% wt/vol NaN_3, 1% vol/vol FCS, incubated with PE conjugated mAb for 25 min at 4°C, and washed twice in ice cold PBS. Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Live cells were gated using forward and side scatter and 10,000 cells were analyzed in each sample.

Biosynthetic Labeling, Solubilization, Immunoprecipitation, and Electrophoresis

For metabolic labeling studies, cells were washed twice in PBS and resuspended at 5×10^6 cells/ml RPMI 1640 without methionine at 37°C for 30 min. After starvation, cells were washed once, resuspended at 5×10^7 cells/ml RPMI 1640 without methionine, and incubated for the period indicated with 500 μCi/ml [^{35}S]methionine (Amersham Laboratories, Amersham, UK). In pulse–chase experiments cells were pulsed for 30 min at 37°C and chased in complete medium for the indicated periods. Cells were washed three times in cold PBS, and solubilized for 30 min at 4°C in lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 150 mM NaCl, 1 mM PMSF, 8 mM iodoacetamide, and 1% digitonin (Sigma Chemical Co., St. Louis, MO). The supernatant obtained after centrifugation for 5 min at 1500 *g* was precleared by incubation with protein A–Sepharose (PA) (Pharmacia LKB Biotechnology, Uppsala, Sweden). For immunoprecipitation, precleared lysate was incubated with 1.0 μg of mAb for 2 h at 4°C. PA was added and the incubation continued for 2 h. The immunoprecipitates were washed five times in lysis buffer, boiled for 5 min with sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol), and subjected to SDS-PAGE on 12% acrylamide gels. Autoradiography of dried gels was performed by using Hyperfilm-MP (Amersham). ^{14}C-labeled proteins from Amersham were used as molecular mass markers.

Cell Surface Iodination

Cells were surface iodinated as previously described (19) and solubilized for 45 min at 4°C in lysis buffer. Immunoprecipitation, electrophoresis, and autoradiography were performed as described above.

Deglycosylation Procedures

For deglycosylation studies, cells were surface iodinated, solubilized, and immunoprecipitated as described. The immunoprecipitates were boiled for 5 min in elution buffer (0.5% SDS, 20 mM Tris-HCl, pH 8.0). The eluate was divided in two parts and treated with either 10 U/ml of *N*-glycanase (Genzyme, Cambridge, MA) or left untreated. After incubation for 18 h at 37°C the eluates were boiled for 5 min in 2× sample buffer and subjected to SDS-PAGE on 12% acrylamide gels.

Tunicamycin Treatment

Cells were biosynthetically labeled as described above in the presence or absence of 1 μg/ml tunicamycin (Boehringer Mannheim, Vedbœk, Denmark).

Results

Prediction of Potential Interaction Sites in the Extracellular Domain of CD3γ from a Computer Model

The EC domain of CD3γ has been suggested to fold as an Ig domain since the residues that are considered to be a hallmark of Ig related sequences are present in the CD3γ EC domain (35, 60). The strategy used to identify potential interaction sites in the EC domain of CD3γ was to search for conserved CD3γ-specific regions that were not shared with other members of the Ig superfamily since a potential interaction site should not include structurally important amino acids. By alignment of CD3γ from human, mouse, rat, and sheep two conserved regions namely amino acid 17–21 and 57–60 were identified (Fig. 1 *A*) and (39). The 17–21 and 57–60 regions are not generally shared between CD3γ and other members of the Ig superfamily except CD3δ in which the 57–60 site, in particular, shows a high degree of conservation (Fig. 1 *A* and reference 35, 60). The 57–60 region was conserved even in the avian CD3 T11.15 chain except from a Lys to Asp substitution at position 57 (Fig. 1 *A*). The mere conservation of these CD3γ(δ) regions between species suggests that they serve an important functional or structural role. However, since these regions are not conserved among other members of the Ig superfamily it is unlikely that they serve a structural role e.g., in stabilizing the native conformation of the assumed EC Ig domain of CD3γ. To examine whether the 17–21 and 57–60 regions could constitute possible interaction sites a computer model of the EC domain of CD3γ was built. According to the computer model, the EC domain of CD3γ folds as an Ig C domain. A secondary structure prediction according to the Kabcsh-Sander algorithm (27), based on the ϕ and ψ angles and hydrogen bonding pattern of the residues in the computer model, confirms two anti-parallel β-sheets characteristic of an Ig C domain. One β-sheet contains three anti-parallel β-strands (*C*, *F*, and *G*) whereas the other contains four such strands (*A*, *B*, *E*, and *D*) (Fig. 2 *A*). Fig. 1 *A* shows the position of these strands in the primary sequence of CD3γ. Secondary structural elements, such as the anti-parallel β-sheets of an

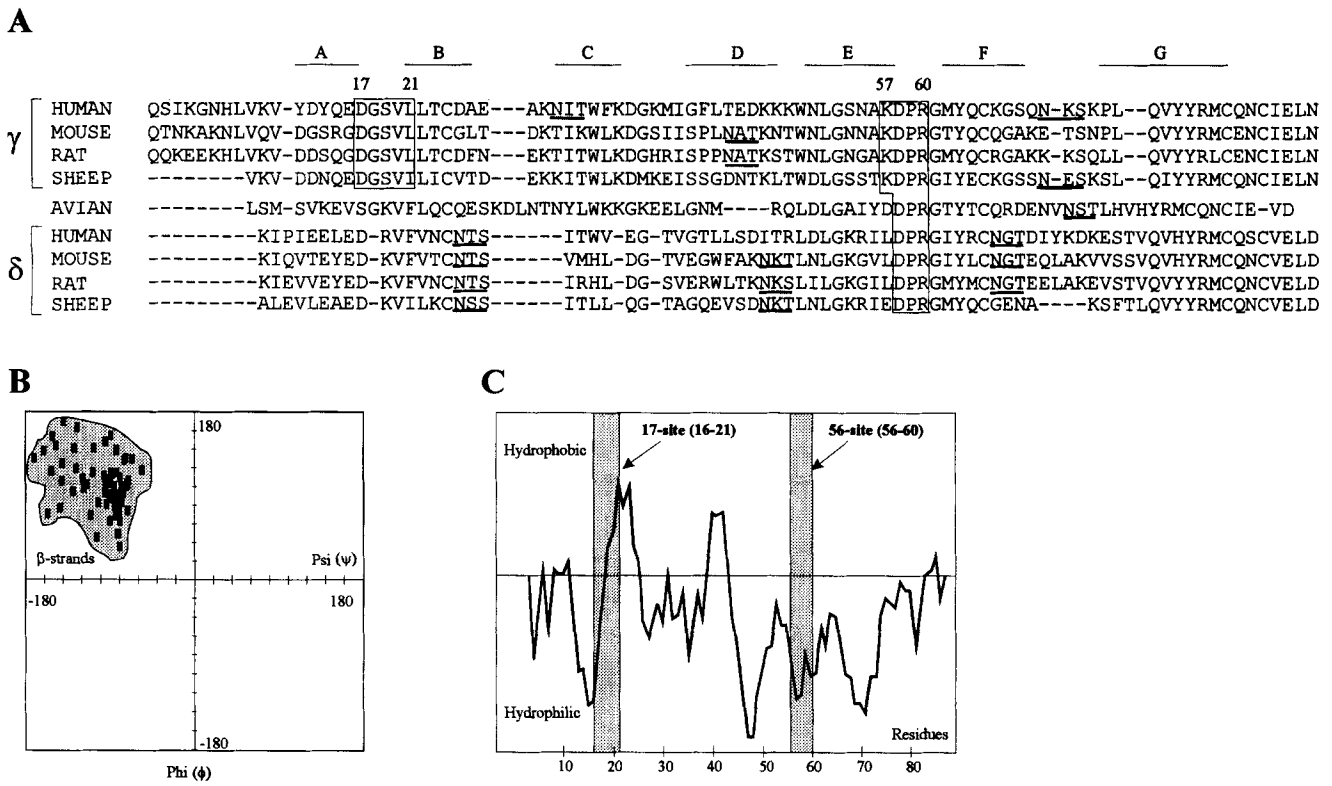

Figure 1. Primary and secondary structure analysis of CD3γ. (*A*) Alignment of the EC domains of CD3γ and CD3δ from human, mouse, rat, and sheep compared to avian CD3. Glycosylation sites are underlined. Boxes denote the two initially identified interaction sites. The position of the β-strands according to the computer model are shown. (*B*) Ramachandran plot of the EC domain of CD3γ. Only residues that according to the computer model are part of the β-strands are shown. Gly residues are omitted. (*C*) Kyte-Doolittle plot of the EC domain of CD3γ. The 17 and 56 sites are shown in grey boxes.

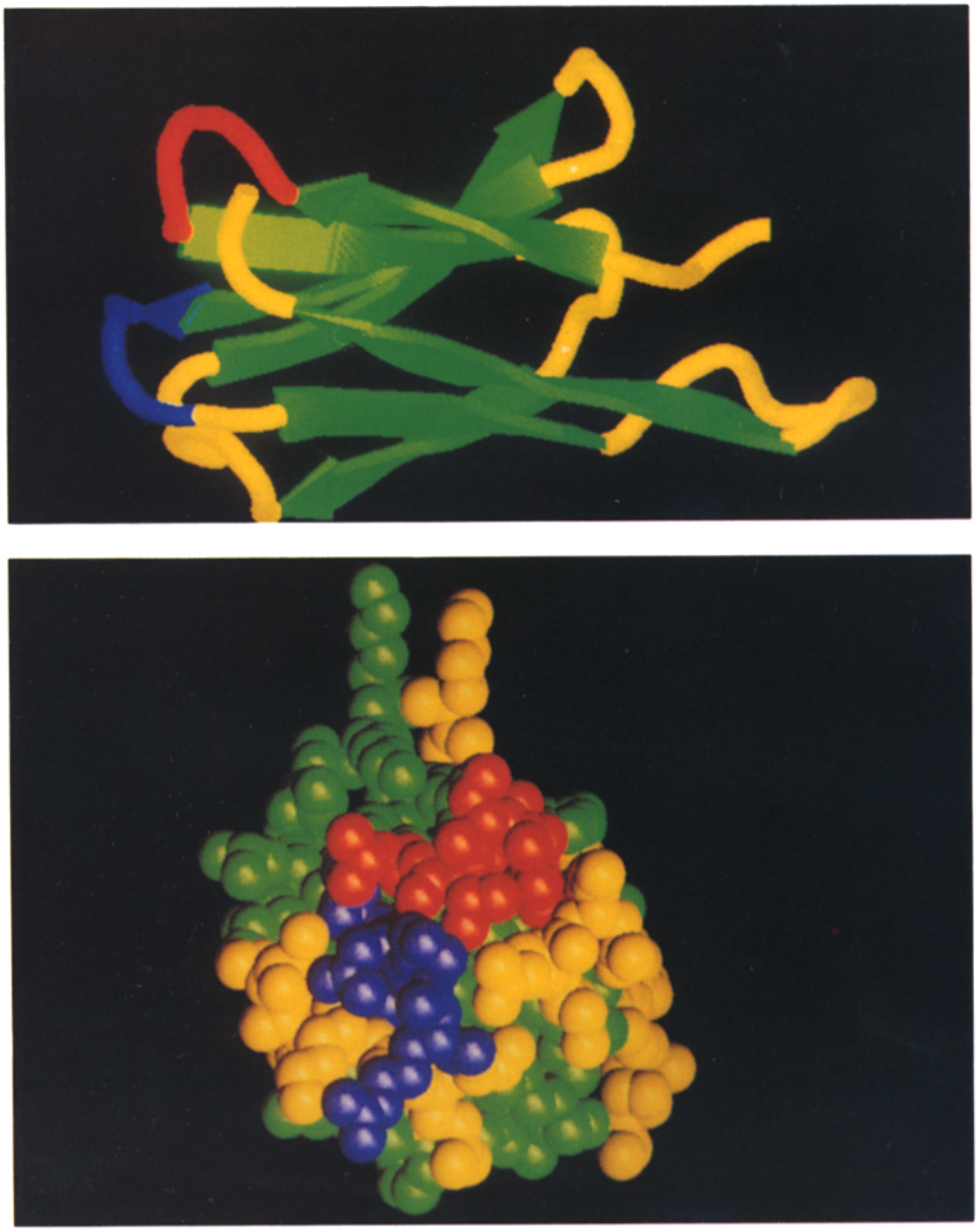

Figure 2. Computer model of the extracellular domain of CD3γ. (*A*) The EC domain of CD3γ. Loops are colored in yellow with the 17–21 and 57–60 regions colored in red and blue, respectively. The arrows (*green*) show the β-strands according to the Kabsh-Sander algorithm (27). (*B*) Space filling model of the EC domain of CD3γ seen from the 16–21 and 56–60 regions. Loops are colored in yellow, strands are colored in green and the two regions 16–21 and 56–60 in red and blue, respectively.

Ig domain, impose restrictions upon the ϕ and ψ angles of its residues (54) unless the residue is a Gly that is less restricted. A Ramachandran plot of the computer model (Fig. 1 *B*) showed that the residues, which according to the model were part of the β-strands, did in fact exhibit ϕ and ψ angles that were within the restricted anti-parallel β-strand area. The Gly residues were omitted in this plot.

According to the secondary structure prediction based on the Kabcsh-Sander algorithm (27) the 17–21 region and the majority of the 57–60 region was found in loops (Figs. 1 *A* and 2). Since loops only have a minor role in maintaining the structure of a protein, this supported the idea that the 17–21 and 57–60 regions did not serve as structurally important regions but could function as interaction sites. Protein-protein interactions usually involve hydrophobic or hydrophilic contacts (25). In a Kyte-Doolittle plot (36) the 17–21 region was found to include part of the most hydrophobic region of the EC domain of CD3γ as well as a hydrophilic region (Fig. 1 *C*). In the computer model the hydrophobic part of the 17–21 region was found to contain two exposed hydrophobic residues (Val 20 and Leu 21). Glu 16 is conserved among human and sheep CD3γ and is exposed according to the computer model. We therefore included Glu 16 in the hydrophilic part of the first conserved region and selected the 16–21 region as a potential interaction site. This region was hereafter denoted the 17 site (Fig. 3).

The 57–60 region was in a Kyte-Doolittle plot (Fig. 1 *C*) found to constitute one of the most hydrophilic regions of the EC domain of CD3γ. This region contains three charged residues (Fig. 1 *A*). These charged amino acids could be involved in salt bridges between CD3γ and another protein of the TCR. Alternatively they could merely interact with water or be involved in intra-molecular salt bridges. However, since neither of these interactions are major contributors to the stability of a protein (17) this

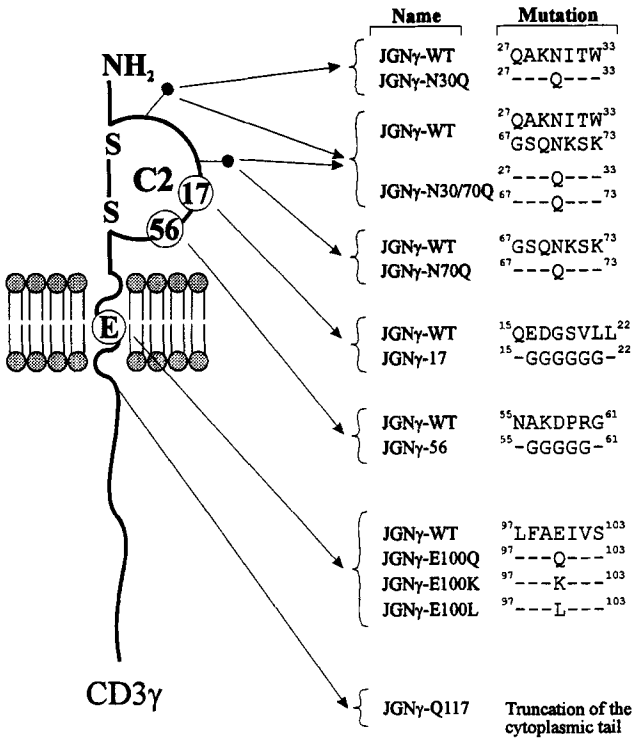

Figure 3. Overview of the transfectants.

would probably not explain the conservation of these residues among CD3γ chains from several species. The computer model showed that two of these charged residues were exposed (Asp 58 and Arg 60) (Fig. 2). Since Ala 56 is conserved among human, mouse, and rat CD3γ and was exposed according to the computer model, the 56-60 region was selected as another potential interaction site. Hereafter this region was denoted the 56 site (Fig. 3).

Role of the 17 and 56 Sites in TCR Assembly and Expression

The production of the CD3γ negative T cell variant JGN (18) allowed us to study the consequences of various mutated CD3γ chains in TCR assembly and expression. JGN cells do not express the TCR at the cell surface but upon transfection of cDNA encoding wild-type CD3γ into JGN cells they become TCR cell surface positive (Fig. 4 *A*). To examine whether the 17 and 56 sites constituted interaction sites, the amino acids 16-21 and 56-60 were replaced with Gly by site-directed mutagenesis in two separate constructs, CD3γ-17 and 56 (Fig. 3). Gly was selected because it is expected to adopt the conformation required for the stability of the secondary structure due to its flexibility with respect to the ϕ and ψ angles (54). Therefore, Gly should not add any strain on the structure. Furthermore, according to the computer model both the 17 and 56 sites

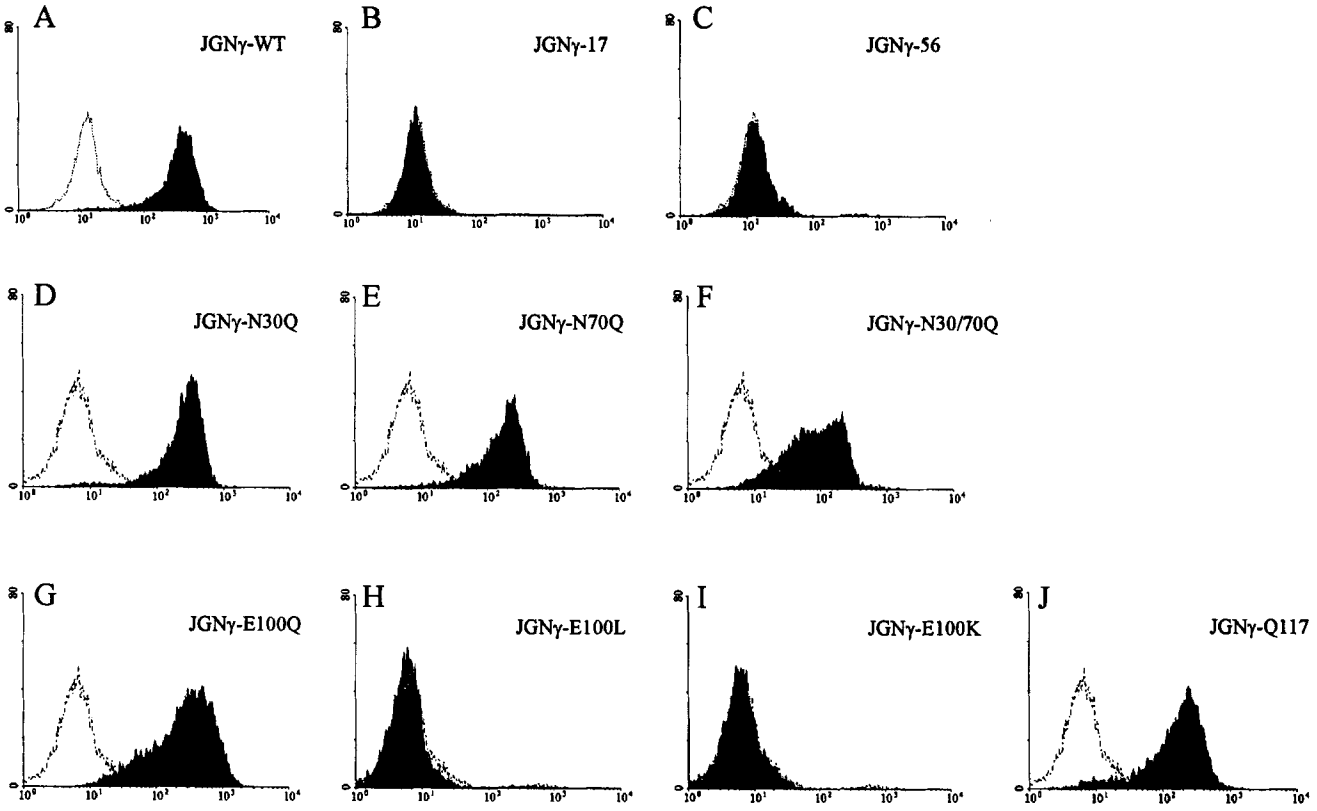

Figure 4. Cell surface expression of the TCR. The parental and transfected JGN cells were stained with anti-CD3ε mAb (*UCHT1*) directly conjugated with PE and subsequently analyzed by flow cytometry. The abscissa represents the fluorescence on a logarithmic scale and the ordinate the relative cell number on a linear scale. The fluorescence histogram of each transfected cell line (*filled histograms*) is compared to the fluorescence histogram of the parental JGN cell line (*open histograms*). The analyzed cell line is indicated in each histogram: JGNγ-WT (*A*), JGNγ-17 (*B*), JGNγ-56 (*C*), JGNγ-N30Q (*D*), JGNγ-N70Q (*E*), JGNγ-N30/70Q (*F*), JGNγ-E100Q (*G*), JGNγ-E100L (*H*), JGNγ-E100K (*I*), and JGNγ-Q117 (*J*).

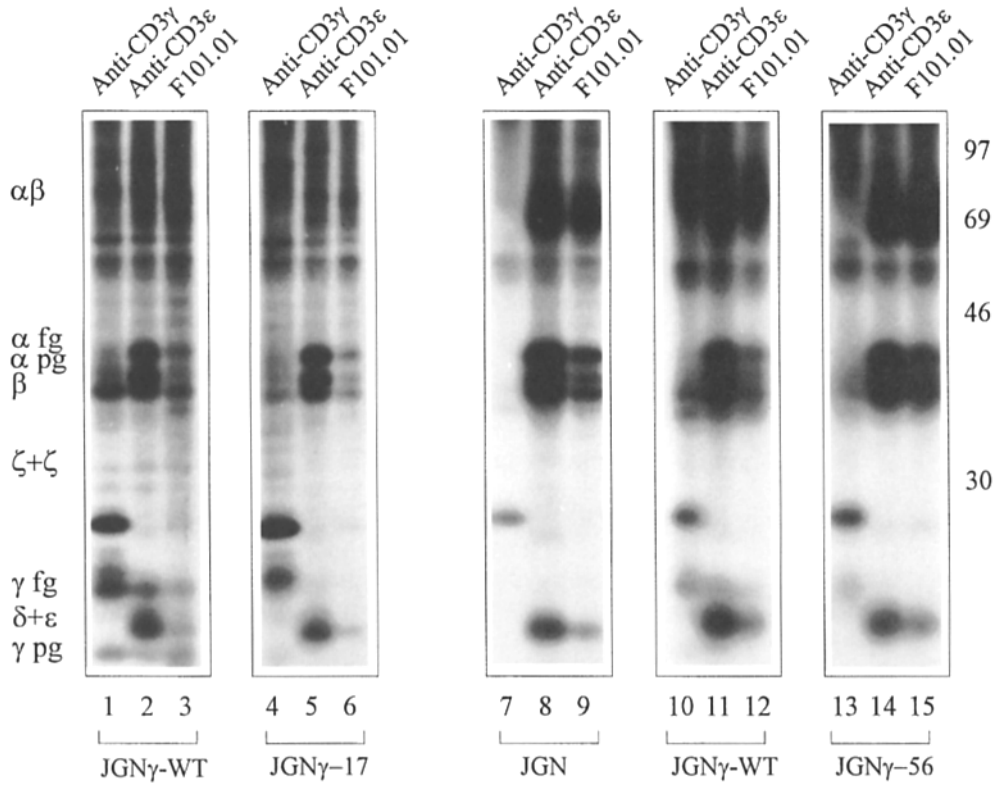

Figure 5. Metabolic labeling and immunoprecipitation. JGNγ-WT (lanes *1–3* and *10–12*), JGNγ-17 (lanes *4–6*), JGN (lanes *7–9*) and JGNγ-56 (lanes *13–15*) cells were metabolically labeled for 30 min, lysed in 1% digitonin lysis buffer, and immunoprecipitated with either anti-CD3γ (lanes *1*, *4*, *7*, *10*, and *13*), anti-CD3ε (lanes *2*, *5*, *8*, *11*, and *14*) or F101.01 mAb (lanes *3*, *6*, *9*, *12*, and *15*). Samples were analyzed by SDS-PAGE under nonreducing conditions on a 12% polyacrylamide gel. The positions of the TCR chains are indicated (*fg*, fully glycosylated; *pg*, partially glycosylated).

constituted loops and loops seem to prefer Gly due to the conformational requirements of the amino acids in a loop region (11). In addition, with respect to the functional consequence of the substitution, we expected that Gly would only make a minor contribution to an interaction, whether this was hydrophilic or hydrophobic, due to its lack of side chain.

The CD3γ-WT, -17, and -56 constructs were separately transfected into JGN cells. Stable clones were selected in medium containing G418 and 30 clones from each of the transfection experiments were subsequently screened for TCR surface expression by FACS analysis. 23 of the JGNγ-WT clones expressed the TCR at the cell surface whereas none of the JGNγ-17 or JGNγ-56 clones expressed the TCR (Fig. 4, *A–C*). To analyze whether CD3γ was synthesized in the transfectants, JGN, JGNγ-WT, JGNγ-17, and JGNγ-56 cells were metabolically labeled, solubilized in lysis buffer containing 1% digitonin, and immunoprecipitated with anti-CD3γ, anti-CD3ε, or F101.01 mAb that only recognizes CD3γε or CD3δε dimers in association with the Ti chains (19). All of the transfectants synthesized a protein at the size of the CD3γ chain (Fig. 5, lanes *1*, *4*, *10*, and *13*) demonstrating that the lack of TCR cell surface expression in JGNγ-17 and JGNγ-56 cells was not due to a lack of the CD3γ chain. In JGNγ-WT cells the anti-CD3ε mAb clearly coprecipitated CD3γ in contrast to JGNγ-17 and JGNγ-56 cells in which CD3γ did not coprecipitate with CD3ε (Fig. 5, lanes *2*, *5*, *11*, and *14*). Immunoprecipitation with F101.01 demonstrated that both CD3γε and CD3δε dimers associated with the Tiαβ chains in JGNγ-WT cells (Fig. 5, lanes *3* and *12*). In contrast, only CD3δε dimers were found associated with the Tiαβ chains in JGNγ-17 and JGNγ-56 cells (Fig. 5, lanes *6* and *15*). These results demonstrated that mutation of the 17 and 56 sites had a pronounced effect on TCR assembly and expression. The formation of CD3γε dimers was markedly decreased in JGNγ-17 and -56 cells as compared with JGNγ-WT and the association between CD3γ and the Tiαβ dimer seemed to be reduced, especially in JGNγ-56 cells. The band with a molecular mass of ~27 kD that was precipitated with the anti-CD3γ mAb (Figs. 5 and 9) was also seen in the CD3γ negative cell line, JGN, (Fig. 5, lane *7*) and was therefore regarded as a protein unrelated to the TCR components.

Role of N-linked Glycosylation in TCR Assembly and Expression

The role of N-linked glycosylation of the TCR chains is unknown. The EC domain of CD3γ contains two sites for N-linked glycosylation, Asn 30 (N30) and Asn 70 (N70) (Fig. 1 *A*). To examine the role of CD3γ glycosylation in TCR assembly and expression three different CD3γ constructs were made by site-directed mutagenesis. In CD3γ-N30Q Gln substituted for Asn 30, in CD3γ-N70Q Gln substituted for Asn 70, and in CD3γ-N30/70Q Gln substituted for both Asn 30 and 70 (Fig. 3). Gln was selected to substitute for Asn because Gln both physically and chemically is similar to Asn and Gln is often seen exchanged with Asn in homologous proteins (14). Therefore, the consequences of the mutations should only be due to the lack of glycosylation. The constructs were separately transfected into JGN cells. Stable clones were selected in medium containing G418 and several clones from each transfection experi-

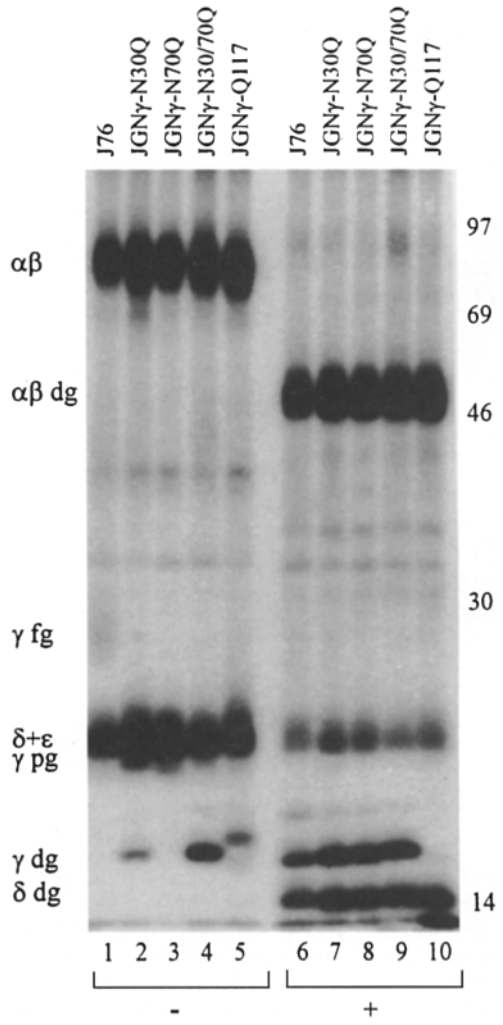

Figure 6. Cell surface labeling. J76 (lanes *1* and *6*), JGNγ-N30Q (lanes *2* and *7*), JGNγ-N70Q (lanes *3* and *8*), JGNγ-N30/70Q (lanes *4* and *9*) and JGNγ-Q117 (lanes *5* and *10*) cells were surface iodinated, solubilized in digitonin lysis buffer and immunoprecipitated with anti-CD3ε mAb. Immunoprecipitates were either left untreated (lanes *1–5*) (−) or were treated with N-glycanase (lanes *6–10*) (+). Samples were analyzed by SDS-PAGE under nonreducing conditions on a 12% polyacrylamide gel. The positions of the TCR chains are indicated (*fg*, fully glycosylated; *pg*, partially glycosylated; *dg*, deglycosylated [non-glycosylated]).

ment were subsequently screened for TCR surface expression by FACS analysis. As demonstrated in Fig. 4, *D–F* all of the constructs allowed TCR cell surface expression. To verify that the transfectants did express the TCR containing the mutated CD3γ chains at the cell surface, J76, JGNγ-N30Q, JGNγ-N70Q, and JGNγ-N30/70Q cells were surface iodinated, solubilized in lysis buffer containing 1% digitonin, and immunoprecipitated with anti-CD3ε mAb. The immunoprecipitates were either treated with *N*-glycanase or left untreated. J76 cells expressed the fully glycosylated wild-type CD3γ with a relative molecular mass of 25–28 kD before treatment with *N*-glycanase and a relative molecular mass of 18 kD after *N*-glycanase treatment (Fig. 6, lanes *1* and *6*). Interestingly, JGNγ-N30Q cells expressed two forms of CD3γ. One was partially glycosylated and had a relative molecular mass of 19–20 kD

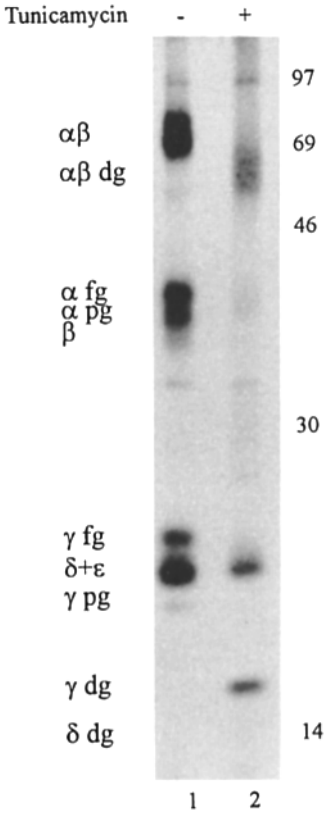

Figure 7. Treatment with tunicamycin. J76 cells were metabolically labeled in the absence (−) or presence (+) of tunicamycin. Subsequently, the cells were lysed in digitonin lysis buffer and immunoprecipitated using an anti-CD3ε antibody. Samples were analyzed under nonreducing conditions by SDS-PAGE on a 12% acrylamide gel. The positions of the TCR chains are indicated (*fg*, fully glycosylated; *pg*, partially glycosylated; *d*, deglycosylated [non-glycosylated]).

whereas the other was not glycosylated and had a molecular mass of 18 kD (Fig. 6, lanes *2* and *7*). JGNγ-N70Q cells only expressed the partially glycosylated form of CD3γ and JGNγ-N30/70Q cells only expressed the nonglycosylated CD3γ as expected (Fig. 6, lanes *3*, *4*, *8*, and *9*).

To further study the role of N-linked glycosylation in the assembly of the TCR, J76 cells were preincubated with tunicamycin, metabolically labeled in the presence of tunicamycin, solubilized in lysis buffer containing 1% digitonin, and subsequently immunoprecipitated with anti-CD3ε mAb. Fig. 7 demonstrates that non-glycosylated CD3γ and Tiαβ dimer coprecipitated with CD3ε. However, the non-glycosylated CD3δ chain did not coprecipitate with the rest of the complex. This indicated that in contrast to CD3γ, the glycosylation of CD3δ (or another protein) is required in the assembly of CD3δ and CD3ε.

To examine whether glycosylation of CD3γ influences its stability in the ER, the JBN cell line, that contains all the TCR chains except Tiβ, was transfected with CD3γ-N30/70Q and stable clones were selected in medium containing G418. The absence of Tiβ ensured that the TCR chains would not escape the ER and the presence of CD3γ-WT allowed comparison of the stability of the nonglycosylated, the fully glycosylated, and the partially glycosylated CD3γ chain. JBN-N30/70Q cells were pulse labeled for 30 min and chased for 0, 1, 2, 6, or 20 h before solubilization in digitonin lysis buffer and immunoprecipitation. Even after a 20-h chase period both the fully and non-glycosylated CD3γ chains were seen (Fig. 8). This indicated that N-linked glycosylation did not influence the stability of the CD3γ. The disappearance of the partially glycosylated CD3γ chain could indicate an instability of this chain, or could merely be due to complete glycosyla-

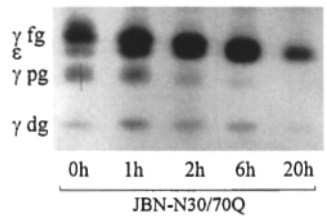

Figure 8. Pulse–chase experiment. JBN-N30/70Q were pulse-labeled for 30 min at 37°C and chased for 0 h, 1 h, 2 h, 6 h, or 20 h. After solubilization in 1% digitonin, immunoprecipitations were performed using an anti-CD3γ antibody (*HMT3.2*). The immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions on a 12% polyacrylamide gel. The positions of the TCR chains are indicated (*fg*, fully glycosylated; *pg*, partially glycosylated; *d*, deglycosylated [non-glycosylated]).

tion of this chain. The observed decrease in molecular mass of the fully glycosylated CD3γ chain during the chase period is probably due to oligosaccharide modifications in the ER (32).

Role of the Transmembrane Charged Amino Acid in TCR Assembly and Expression

To examine the role of the charged amino acid Glu (E100) in the TM domain of CD3γ, three different constructs were made in which Gln, Leu, and Lys substituted for Glu, respectively (Fig. 3). The constructs were separately transfected into JGN cells. Stable clones were selected in medium containing G418 and 30 clones from each of the transfection experiments were subsequently screened for TCR surface expression by FACS analysis. 21 of the JGNγ-E100Q clones expressed the TCR at the cell surface whereas none of the JGNγ-E100L or JGNγ-E100K clones expressed the TCR (Fig. 4, *G–I*). To analyze whether a CD3γ chain was synthesized in the transfectants, JGNγ-WT, JGNγ-E100Q, JGNγ-E100L or JGNγ-E100K cells were metabolically labeled, solubilized in lysis buffer containing 1% digitonin, and immunoprecipitated with anti-CD3γ, anti-CD3ε, or F101.01 mAb. All of the transfectants synthesized a protein at the size of the wild-type CD3γ chain, except for JGNγ-E100K cells in which the mobility of the CD3γ-E100K chain seemed to be markedly influenced by the mutation as the mutated CD3γ chain comigrated with the CD3δ and ε band (data not shown). In JGNγ-WT and JGNγ-E100Q cells the anti-CD3γ mAb coprecipitated the Tiαβ dimer, the single Tiβ and α chains, the ζ_2 dimer and the CD3ε chain (Fig. 9, lanes *1* and *2*). In contrast, in JGNγ-E100L the anti-CD3γ mAb did not coprecipitate the ζ_2 dimer and the single Tiβ and α chains, and only faint bands of Tiαβ dimer were seen as compared with JGNγ-WT and JGNγ-E100Q cells. Only the CD3ε chain was coprecipitated as strongly with CD3γ in JGNγ-E100L cells as in JGNγ-WT and JGNγ-E100Q cells (Fig. 9, lanes *1–3*). The anti-CD3ε mAb coprecipitated CD3γ with similar efficiency from all three cell lines (Fig. 9, lanes *4–6*). The F101.01 mAb precipitated both CD3γε and CD3δε dimers together with the rest of the TCR from JGNγ-WT and JGNγ-E100Q cells but did not precipitate CD3γε dimers from JGNγ-E100L cells (Fig. 9, lanes *7–9*). In contrast to JGNγ-WT and JGNγ-E100Q cells the ζ_2 dimer was not coprecipitated in JGNγ-E100L cells with any of the mAbs tested further supporting the findings that the TCR was not properly assembled in JGNγ-E100L cells. These experiments demonstrated

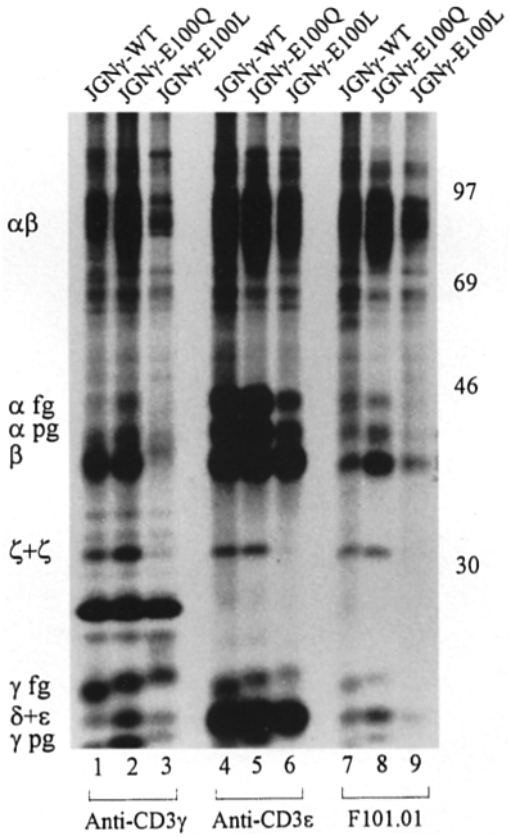

Figure 9. Metabolic labeling and immunoprecipitation. JGNγ-WT (lanes *1*, *4*, and *7*), JGNγ-E100Q (lanes *2*, *5*, and *8*), and JGNγ-E100L (lanes *3*, *6*, and *9*) cells were metabolically labeled for 90 min, lysed in digitonin lysis buffer, and then immunoprecipitated using either anti-CD3γ (lanes *1–3*), anti-CD3ε (lanes *4–6*), or F101.01 mAb (lanes *7–9*). Samples were analyzed by SDS-PAGE under nonreducing conditions on a 12% polyacrylamide gel. The positions of the TCR chains are indicated (*fg*, fully glycosylated; *pg*, partially glycosylated).

that the chemical and physical properties of the TM Glu in CD3γ play an important role in TCR assembly and expression at the cell surface.

Role of the Cytoplasmic Domain in TCR Assembly and Expression

To examine the role of the CY domain of CD3γ in TCR assembly and expression the construct CD3γ-Q117^{stop} was made, in which the entire CY tail of CD3γ was deleted (Fig. 3). The construct was transfected into JGN cells, stable clones were selected in medium containing G418 and subsequently screened for TCR surface expression by FACS analysis. As demonstrated in Fig. 4 *J*, truncation of the CY tail of CD3γ did not impair TCR expression. To verify that CD3γ-Q117^{stop} did express the TCR containing the tail-less CD3γ chain at the cell surface, cells were surface iodinated, solubilized in lysis buffer containing 1% digitonin, and then immunoprecipitated with anti-CD3ε mAb. The immunoprecipitates were either treated with *N*-glycanase or left untreated. As demonstrated in Fig. 6, lanes *5* and *10*, JGNγ-Q117 cells did express the TCR containing the truncated CD3γ chain at the cell surface.

Discussion

The Extracellular Domain

Although the majority of studies describing interactions of the TCR subunits have concentrated on the charged amino acids in the TM domains (1, 3, 13, 23, 26, 42) a role of the EC C domains of Tiα and β for complete assembly of the TCR in T cells has been described (9, 21). These studies describe point mutations of conserved amino acids that caused conformational changes of the Ti C domains and abolished correct TCR assembly and expression (9, 21). Recently, other experiments have also indicated a role for the EC domain of the TCR chains in the assembly of the TCR complex (41, 59). Cotransfecting pairs of CD3 chains and chimeric Tiβ-IL2 receptor α chains into COS cells indicated that the association between CD3γ, δ, or ε and Tiβ was stabilized by the ECC domain of the Tiβ chain (59). Another experiment demonstrated that assembly of Tiβ with Tiα or CD3ε involved the extracellular domains of these chains (41). However, it should be noted that these results were obtained by cotransfecting COS cells with different TCR chains and chimeric proteins. These experiments reflect the ability of isolated TCR chains to interact with each other and do not necessarily represent the interactions that take place in the fully assembled TCR in T cells. We have recently demonstrated that the EC domain of CD3δ can not substitute for the EC domain of CD3γ in assembly of the complete TCR in T cells (58). This indicated that the EC domain of CD3γ might have a role in TCR assembly and lead us to search for potential interaction sites in this domain. The search was made according to the following criteria: (*a*) the site(s) should be conserved among CD3γ chains but not among other members of the Ig superfamily since general conserved residues most probably have a structural role; (*b*) the site(s) should include a hydrophilic/hydrophobic region since protein-protein interactions often involve hydrophilic/hydrophobic contacts; (*c*) the site(s) should contain exposed hydrophilic/hydrophobic residues that could be directly involved in an interaction; and (*d*) the site(s) should probably be found in loop regions since such regions have a minor structural role but could have an important functional role. Based on alignment studies (Fig. 1 *A* and reference 39), a Kyte-Doolittle plot (Fig. 1 *C*) and a computer model of the extracellular domain of CD3γ (Fig. 2) two sites were found to fulfill these criteria, namely the 17 and 56 site. The present study demonstrated that both sites were required for proper TCR assembly and cell surface expression. The mutagenized CD3γ (CD3γ-17 or -56) associated weakly, if at all, with CD3ε and binding to the Tiαβ dimer seemed to be reduced. In contrast, the association to the single Tiβ seemed to be preserved. It is interesting that both the 17 and 56 site mutations seemed to have almost identical effects on TCR assembly. This indicated that both sites could be part of the same binding site to CD3ε. According to the computer model this is plausible as the 17 and 56 sites are in close contact to each other (Fig. 2 *B*). A more detailed analysis involving single point mutations is required to precisely define the binding site to CD3ε. We can not exclude the possibility that the mutations affected the general conformation of the EC domain of CD3γ that might be supported by the observation that the partially glycosylated form of CD3γ was not as clearly seen in JGNγ-17 and JGNγ-56 cells as in JGNγ-WT cells. However, we can conclude that the EC domain of CD3γ is involved in TCR assembly. The preserved binding between the mutated CD3γ in JGNγ-17 or -56 cells and the single Tiβ indicated that pair-wise interactions between CD3γ and Tiβ is not dependent on the 17 and 56 sites. In contrast, the association of CD3γ with CD3ε seems to require the 17 and 56 sites. Both the 17 and 56 sites are partially conserved between CD3γ and CD3δ chains from human, mouse, rat, and sheep. Therefore, it could be speculated that these sites serve similar functions in CD3γ and CD3δ. Experiments have shown that CD3γε and CD3δε dimers can be formed independently of other TCR chains (4, 18) and that CD3γ and CD3δ compete for the binding to CD3ε (18). This suggests that CD3γ and δ each contain a binding site to CD3ε and furthermore share a binding site on CD3ε. The binding site to CD3ε could be the 17 and 56 sites in both CD3γ and CD3δ.

Oligosaccharides modify the local structure and overall dynamics of the proteins to which they are attached. They may alter the functional activity of a protein, they may be involved in molecular recognition events, and they may be involved in signal transduction (reviewed in reference 53). Recently a role of oligosaccharides in TCR assembly has been described. Persistence of glucose residues on N-linked oligosaccharides disrupted the associations of the chaperone calnexin with both the Tiα and β, and furthermore restricted the ability of the Tiα and β to form heterodimers (28). In this study we demonstrated that N-linked glycosylation of CD3γ is not required for TCR assembly and expression. By combining the results from the computer model and the alignment of human, mouse, rat, and sheep CD3γ we found that the N-glycosylation sites in all species were located in other regions of CD3γ than the putative interaction sites 17 and 56. Furthermore, the CD3γ N-glycosylation sites were not conserved with respect to their position. This lack of conservation suggested that the N-glycosylation of CD3γ is not involved in intermolecular interactions between the TCR subunits. In addition, since the non-glycosylated CD3γ chain was able to function in TCR assembly and expression, this indicated that glycosylation did not have a significant influence on the native conformation of CD3γ. The glycosylation of the TCR chains could, apart from TCR assembly (28), be involved in molecular recognition events at the cell surface of T cells and/or in signal transduction. Another potential role for glycosylation could be in the degradation of TCR chains in the ER (5). However, our experiments indicated that the stability of CD3γ in the ER was not affected by glycosylation.

It is interesting that JGNγ-N30Q cells in addition to the partially glycosylated CD3γ also expressed the non-glycosylated CD3γ chain at the cell surface. This suggested that the efficient glycosylation of Asn 70 to some extent is dependent on the glycosylation of Asn 30. The glycosylation of Asn 30 might cause a conformational change, early in the folding process of CD3γ, which is required for efficient glycosylation of Asn 70. Experiments performed with tunicamycin showed that the non-glycosylated CD3δ, in contrast to the other TCR chains, was not coprecipitated with CD3ε. This indicated a role of CD3δ glycosylation in TCR

assembly either directly by interacting with other TCR chains or indirectly by influencing the structure of CD3δ. Alternatively, an unknown protein involved in CD3δε dimer formation could be affected. The N-linked glycosylation sites of CD3δ are located differently than the glycosylation sites of CD3γ (Fig. 1 *A*). In CD3δ three (mouse and rat) or two (human and sheep) glycosylation sites are found. In contrast to CD3γ these sites show a high degree of conservation. Interestingly, two glycosylation sites are located just carboxy-terminal of the cysteines responsible for the intramolecular disulfide bond between the β-strands B and F. The first site is conserved among all four species whereas the second site is conserved among CD3δ from human, mouse, and rat (Fig. 1 *A*). The β-strands B and F are the most conserved regions in members of the Ig superfamily and it is likely that attachment of oligosaccharide in the center of these strands, next to the most conserved amino acid in all members of the Ig superfamily, may have an important influence on the conformation of CD3δ. Further studies are in progress to study the role of N-linked glycosylation in TCR assembly.

The Transmembrane Domain

Cross-binding experiments have demonstrated a close contact between CD3γ and Tiβ at the T cell surface (6, 31, 52). Other experiments have indicated that this association could involve the TM charged residues of Tiβ and CD3γ since point mutations of these amino acids abrogated TCR expression in T cells (1) and CD3-Ti association in COS cells (23), respectively. This is in agreement with our results which demonstrated that the TM charged residue of CD3γ is involved in TCR assembly in T cells, probably through a direct interaction, in the form of a salt bridge, with the basic TM residue in Tiβ. Mutations of Glu 100 in the TM domain of CD3γ demonstrated that Glu or Gln was required for complete assembly and expression of the TCR. If Leu or Lys substituted for Glu 100 the TCR was not expressed at the cell surface. The presence of CD3ε in the anti-CD3γ precipitate and CD3γ in the anti-CD3ε precipitate demonstrated that CD3γε dimers were formed in JGNγ-E100L cells. However, in these cells the Tiβ and the Tiαβ bands were markedly decreased in the anti-CD3γ precipitate as compared to JGNγ-WT cells. The lack of CD3γ in the F101.01 precipitate further showed that CD3γ did not associate with Tiαβ in contrast to CD3γ in JGNγ-WT cells. This demonstrated that Glu 100 in the TM domain of CD3γ is not involved in the association with the CD3ε chain but is required for the association between CD3γε dimers and Tiαβ dimers probably through binding to Tiβ. Changing Glu to Gln did not abolish TCR assembly and expression. Since Gln both chemically and physically resembles Glu this indicated that the common properties of these amino acids in forming hydrogen bonds or salt bridges are directly involved in TCR assembly. Such a hydrogen bond or salt bridge should be abrogated if Glu 100 was changed to a hydrogen donor such as Lys or to a nonpolar amino acid such as Leu. The lack of TCR surface expression in the JGNγ-E100K and JGNγ-E100L transfectants supported this hypothesis.

It seems likely that the TM charged amino acids of the TCR subunits have at least two roles: they are involved in TCR assembly in the ER by stabilizing the interaction between the TCR chains and they are involved in regulation of the assembly process by inducing degradation of TCR chains that are in excess or TCR chains which, for some reason, are not able to function properly in the assembly process. The observation that the bands corresponding to partially glycosylated CD3γ and CD3ε were more intense in JGNγ-E100Q cells than in JGNγ-WT cells could suggest that the E100Q mutation had a stabilizing effect on CD3γ.

The presence of nine charged amino acids in the transmembrane region of the TCR complex is puzzling since this gives a net charge of −3 provided that the TCR only contains one Tiαβ dimer. Whether the TCR contains one or two Tiαβ dimers is not fully known. However, recent experiments have indicated that only one Tiαβ dimer is included in each TCR (24, 34, 46, 47). The BCR has a net charge of −4 and the Fc receptors, FcγRIIIA and FcεRI, each have a net charge of −3 (40). The reason for this lack of equilibration of the TM region of the TCR, BCR, and FcR is not known, but it could be suggested that these receptors are equilibrated by the presence of a yet unknown membrane protein(s). Recently it has been found that the murine BCR (mIgD) is associated with two proteins, BAP29 and BAP31, which could equilibrate the transmembrane region of the BCR complex (29).

The Cytoplasmic Domain

It has previously been demonstrated that deletion of the CY domain of either CD3ε (51), CD3δ (7), or ζ(57) does not prevent assembly and cell surface expression of the TCR. In the present paper we showed that the CY domain of CD3γ is not required for TCR assembly and expression. These observations support the hypothesis that whereas the EC and TM domains of the TCR are required for TCR assembly, expression, and recognition, the CY domains are mainly responsible for signal transduction (38, 56, 57) and internalization of the TCR (16).

In conclusion, based on the results from the present studies on TCR assembly in T cells we have demonstrated that (*a*) the association between CD3γ and CD3ε requires specific interaction sites in the EC domain of CD3γ but is independent of the charged amino acids in the TM domain of CD3γ, (*b*) the association between CD3γ and Tiβ requires the charged amino acid in the TM domain of CD3γ (Glu) or an amino acid with similar chemical and physical properties (Gln), and (*c*) the association between CD3γ and Tiαβ is influenced by the ability of CD3γ to assemble with Tiβ and to a lesser degree with CD3ε indicating a hierarchy in the TCR assembly process. Finally, glycosylation of CD3γ does not seem to influence the stability of CD3γ or the assembly of the TCR.

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