The transmembrane domain of T cell receptor (TCR) β contains a conserved immunoreceptor tyrosine-based activation-like motif consisting of a duplicated XYY sequence. The motif is also present in TCRγ, the equivalent chain to TCRβ in γδ T lymphocytes but is absent in TCRα and TCRδ. To determine the putative role of this sequence in TCR-CD3 complex assembly and signal transduction, a TCRβ chain cDNA was mutated in the C-terminal tyrosine of the motif, cloned in an expression vector, and transfected into TCRβ-negative Jurkat cells. Transfectants of the mutated chain (MUT) expressed, on average, much less TCR-CD3 complex on the membrane than wild type TCRβ transfectants. Radiolabeling experiments suggested that the mutation caused a loose association of the CD3ζ chain resulting in a defective assembly. However, stimulation of high TCR-CD3 expressing wild type and MUT clones with monoclonal antibodies and Staphylococcus aureus enterotoxin B resulted in similar levels of CD25 and CD69 expression, interleukin-2 secretion, and TCR-CD3 complex down-regulation. By contrast, MUT cells were clearly resistant to activation-induced cell death, and they did not express CD95-ligand upon activation. These results suggest a differentiated intracellular signaling pathway leading to apoptosis in which Tyr-TM11 of the immunoreceptor tyrosine-based activation motif-like motif and CD3ζ appear to be involved.

The T cell antigen receptor-CD3 complex (TCR-CD3) is formed by a clonotypic heterodimer (αβ or γδ), which provides ligand specificity, non-covalently linked to at least four invariant chains (CD3ε, -γ, -δ, and -ζ) (for review see Refs. 1–3). Assembly occurs by pairwise interactions (4), and as a result, the TCR-CD3 complex is formed by four dimeric components as follows: (a) clonotypic TCRα and TCRβ chains that are covalently linked via a single extracellular disulfide bond; (b) the non-covalent CD3ζγε dimer; (c) the non-covalent CD3ζδε dimer; and (d) a disulfide-linked ζ family dimer consisting of any of the five defined members of this family, ζ, η, θ, ι, and the γ chain of the high affinity Fcε receptor (5). In addition to these interactions, stable pairwise associations can also be observed between single clonotypic and CD3 chains (4, 6, 7). Nevertheless, the CD3ζ dimer will only assemble in the complex if all the other subunits are present, thus explaining why CD3ζ is the last component to be integrated into the TCR-CD3 complex during assembly (8–10). The stoichiometry, as well as the possible formation of alternative TCR-CD3 complexes, is still a question of debate.

The ability of antigen receptors to transduce signals to multiple biochemical cascades is the central event of immune cell activation (11). Engagement of the multicomponent TCR-CD3 complex with its antigen/MHC ligand, agonist mAbs, or superantigens results in several biochemical processes critical for the functional activation of T lymphocytes, including cellular proliferation, cell differentiation, and programmed cell death. Using chimeric molecules and reconstituted receptors, several reports have provided evidence for a redundant signal transduction domain present in the CD3 chains (12). This domain, which contains a pair of XXXI/sequences (where X corresponds to a variable residue), is known as antigen recognition activation motif, activation receptor homology-1, tyrosine-based activation motif, or immunoreceptor tyrosine-based activation motif (ITAM) (13). This sequence, which is triplicated in ζ and present as a single copy in each of the other CD3 chains, has an important role in coupling the TCR-CD3-mediated signaling to intracellular signal transduction molecules (14). However, how the TCRαβ clonotypic receptor is able to deliver signaling events to the other chains of the complex after its stimulation by an antigen remains to be solved. Due to the short length of the cytoplasmic tails and to the apparent lack of inherent signaling activity of both clonotypic chains, it is assumed that the TCR heterodimer transmits the signals produced upon antigen binding through the CD3 chains. TCR and CD3 domains involved in TCR-CD3 complex assembly could also be good candidates to mediate interactions required for signal transduction. In this regard, transmembrane domains of the TCR and CD3 chains have been shown to play a crucial role in assembly, partly due to charge neutralization between the basic residues of TCRβ, TCRα (or TCRγ and TCRδ), and the acidic residues of each one of the CD3 chains (1–3). However, additional residues must participate to add specificity to the interaction.
**CD3ζ Involvement in TCR-CD3-mediated Apoptosis**

The location in the transmembrane region of TCRζ of a conserved ITAM-like motif, which is also present in TCRγ but not in TCRα or TCRδ, prompted us to search for the role this sequence could play in assembly, surface expression, and function of the TCR-CD3 complex, as a possible first signal transducer after TCR stimulation. Although clones that expressed high levels of the TCR-CD3 complex on the cell surface were obtained, the mutation of the C-terminal tyrosine of the ITAM-like motif in TCRζ resulted in an impaired CD3ζ chain assembly. Surprisingly, stimulation of MUT clones through the TCR-CD3 complex resulted specifically in defective induction of programmed cell death, whereas other activation read outs were normal, including IL-2 secretion, IL-2R expression and down-regulation of the TCR complex. A 30418

**31.13 is a TCRζ-negative Jurkat mutant kindly provided by Dr. A. Alcover (Institut Pasteur, Paris) (15).**

**UCHT1, a CD3ε- and CD3ζ-specific mAb, was kindly provided by Dr. P. Beverley (Imperial Cancer Research Fund, London). JOVI-3, a mAb anti-β/γ kindly provided by Dr. M. Owen (Imperial Cancer Research Fund, London). SP34, a CD3ζ chain mAb, was a gift from Dr. C. Terhorst (Beth Israel Hospital, Boston). N39, a polyclonal anti-CD3ζ antibody was kindly provided by Dr. J. Sancho (Instituto López Neyra, Granada, Spain). TPI/55, a mAb anti-CD69, was provided by Dr. F. Sanchez-Madrid (Hospital de la Princesa, Madrid). MAR108, a mAb anti-CD25, was provided by M. Lopez-Botet (Hospital de la Princesa, Madrid). JR2, an anti-TCR Vβ8 mAb, was obtained from Pharmingen (San Diego, CA). Anti-human CD95 mAbs, CH-11 and DX2, were from Upstate Biotechnology Inc. (Lake Placid, NY) and Pharmingen (San Diego, CA), respectively. FITC-conjugated antibodies specific for mouse ιgs and human CD3 were purchased from Southern Biotechnology (Birmingham, AL).

**PCR Procedures and DNA Cloning**

The HA1.7 β chain cDNA (Vβ3) was a gift from Dr. M. Owen (EMBL/GenBank accession number X63456). It was derived from the HA1.7 human CD4+ T cell clone specific for the influenza hemagglutinin (HA) peptide 307-319 in the context of HLA-DR1. For cloning into the pSRα expression vector, XhoI and BamHI restriction sites were introduced at the 5'- and 3'-ends, respectively, of the HA1.7 β chain cDNA. Full wt cDNA was PCR-amplified by using, as amplification primers, the 5′-sense oligonucleotide CCCCTGAGCGATGTTGGGATTACGGCTC (oligo 1), which included the XhoI site, and the 3′-antisense oligonucleotide GGGGATCCTGCAGCCTGTTGTTG (oligo 4), which included the BamHI site.

The construction of the full MUT cDNA involved the following steps. 1) Generation of a PCR-amplified product from the 5′-end of the molecule to the nucleotide 892. Mutations at positions 881 and 882 were introduced by using the 3′-antisense oligonucleotide CGACACAGCAAGCGGGCCG (oligo 2) which included the changes (underlined and boldface) A/Tand T/C to produce the desired Tyr/Leu change. Oligo 1 was used as sense primer for the amplification. 2) Generation of a PCR-amplified product from position 872 to the 3′-end of the cDNA. It was carried out by using the oligonucleotide GCCACCTGGCTTGTTGCT (oligo 3) as 5′-sense primer which is complementary to oligo 2, and the oligo 4 previously described as 3′-antisense primer. 3) Generation of the full cDNA MUT. It was achieved by mixing purified PCR products derived from steps 1 and 2, taking into account that they have complementary tails, and performing a PCR with oligo 1 and oligo 4 as amplification primers.

In all cases, 25 amplification cycles were performed, each consisting of 1 min at 94°C, 2 min at 55°C, 2 min at 72°C, and a single final extension of 5 min at 72°C. Full PCR-derived XhoI/BamHI-tailed cDNAs, WT and MUT, were isolated, cloned into pSRα expression vector previously digested with XhoI and BamHI, and fully sequenced in Sequengel gels (National Diagnostics, Hesse, UK) by the Sequenase (U.S. Biochemical Corp.) method.

**Transfection, Selection, and Surface Expression Analysis**

DNA-mediated gene transfer into 31.13 cells was accomplished by electroporation. 5 × 10⁶ cells were mixed with 40 μg of DNA in a sterile disposable cuvette (Bio-Rad) in a volume of 0.8 ml of RPMI 1640 and electroporated in a Bio-Rad gene pulser unit at 250 V and 960 microfarads. After electroporation, the cells were immediately resuspended in 10 ml of RPMI 1640 (Bio-Whitaker) containing 10% fetal calf serum. Forty-eight hours later, the cells were plated in 96-well flat bottom plates at 2 × 10⁵ cells/well in selective medium containing 1 ng/ml G418 (Sigma). After 3–4 weeks, the growing cells were screened by flow cytometry for surface expression of CD3 (UCHT1), Vγ3 (JOVI-3), and Vp8 (JR2).

**Flow Cytometry**

10⁵–10⁶ cells were incubated on ice for 30 min with a specific mAb (2–4 μg/ml) in PBS, washed, and incubated for additional 30 min with FITC-conjugated anti-mouse IgG antibody. After washing, the cells were analyzed in a flow cytometer (EPICS-XL MCL, Coulter).

**Cell Viability Assays—** 10⁵ cells/well were stimulated with soluble UCHT1 or JOVI-3 at concentrations ranging from 0.5 to 5 μg/ml in the presence of 10 ng/ml PMA. After 24 h, 100 μl of culture supernatant were removed from each well and frozen to ensure that no viable cells remained. The IL-2 content of these supernatants was determined in a CTLL2 proliferation assay. CTLL2 were seeded at 10⁵ cells/well in a 1:2 dilution of the culture supernatants, and 24 h later the cells were pulsed for 6 h with 1 μCi of [³H]thymidine (Amersham Corp., Buckinghamshire, UK) per well. Cells were harvested, and [³H]thymidine incorporation was determined by liquid scintillation counting.

**Cell Viability Assays—** 2 × 10⁵ cells/well were stimulated with plastic-bound mAbs UCHT1, JOVI-3, or soluble SEB at different concentrations. After 48 h, viable and dead cells were counted in a hemocytometer in the presence of 0.25% trypan blue. Antibody-coated plastic wells were prepared by overnight incubation of 96-well plates (Costar, Cambridge, MA) with various concentrations of purified mAb in PBS at 4°C. Down-regulation of CD3—2.5 × 10⁵ cells/well were incubated at 37°C for 20 h with different concentrations of soluble SEB or for 4 h with 10 μg/ml JOVI-3. Cells were washed, stained with a FITC-conjugated CD3 antibody, and analyzed by flow cytometry.

**Flow Cytometry Analysis of DNA Degradation—** 2 × 10⁵ cells/well were stimulated for 48 h with soluble SEB, ranging from 1 to 100 μg/ml, or with anti-CD95 mAb at 500 ng/ml. They were then harvested from culture and permeabilized in 500 μl of 100 μg/ml propidium iodide (Sigma), 0.05% Nonidet P-40, 10 μg/ml RNase, in PBS. Alternatively, cells were stimulated with 1 μg Act2187 plus 15 ng/ml PMA for 24 h. After vortexing, samples were allowed to equilibrate at 4°C in the dark for at least 1 h before being analyzed. Propidium iodide fluorescence analysis was performed by flow cytometry (EPICS-XL MCL, Coulter).

**Metabolic Labeling and Immunoprecipitation**

20 × 10⁶ cells/ml were washed twice with Dulbecco's modified Eagle's medium and incubated for 4 h in Dulbecco's modified Eagle's medium without cytoine and methylion in the presence of 125I-seliotidine (0.5 mCi/m). After labeling, the cells were lysed in 1% Brij 96 (Sigma) lysis buffer containing protease inhibitors. The lysates were pre-cleared twice with preimmune serum and protein A-Sepharose beads. Immunoprecipitation was carried out using specific antibodies pre-bound to protein A-Sepharose beads as described (16).

**Cell Surface Radioliodination**

To enhance the detection of ζ, the cells were pretreated with the water-soluble Bolton-Hunter reagent before radioliodination. Briefly, 20 × 10⁶ cells were washed with PBS, resuspended in 1 ml of PBS plus 200 ng/ml of sulfosuccinimidyl-3-(4-hydroxyphenyl)-proprionate (Pierce), and incubated at room temperature for 30 min. The reaction was stopped by diluting the cells with 10 ml of 10 mM L-lysine in PBS. The cells were centrifuged and 125I-labeled by the lactoperoxidase method and subsequently lysed and immunoprecipitated as above.

**Northern Blot Analysis**

Total RNAs (15–20 μg/lane) were run on 1.1% agarose-formaldehyde gels, transferred to nylon membranes (GeneScreen, DuPont NEN), prehybridized, and hybridized with 32P-dextran sulfate, 1% SDS, and 100 μg/ml salmon sperm DNA. For hybridization, 2 × 10⁶ cpm/ml of random priming or nick translation 32P-labeled probe were added. The specific human Vβ3 gene segment probe was PCR-derived from the HA1.7 β chain cDNA by using oligo 1 (see above) as sense primer and the oligonucleotide 5′-ATATG-AGAAAATAGTACG as antisense primer. Full-length HA1.7 TCRζ
cDNA (17) was used as the TCRα probe. A 486-base pair fragment derived by PCR from murine CD95L cDNA (18) was a gift from A. Ortiz (Fundación Jiménez Díaz, Madrid). Blots were stripped and subsequently rehybridized with other probes, including 28S probe to account for RNA loading variations. Exposed films were scanned with a densitometer (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Expression of a TCR-β Chain Mutated in a Transmembrane Tyrosine Residue Results in Low TCR/CD3 Expression—The comparison between the amino acid sequences of the transmembrane domains of TCRβ and TCRγ from different species have been aligned showing the conservation of the double YXXL/I motif. The corresponding sequences in human TCRα and TCRβ are also shown for comparison. The tyrosine and leucine residues in the double YXXL/I motif have been boxed. Dashes represent conserved amino acids. Asterisks indicate the positions of the transmembrane lysine and arginine residues.

Fig. 1. An ITAM-like motif is present in the transmembrane domains of TCRβ and TCRγ chains. Amino acid sequences (in one-letter code) corresponding to the transmembrane domains of TCRβ and TCRγ from different species have been aligned showing the conservation of the double YXXL/I motif. The corresponding sequences in human TCRα and TCRβ are also shown for comparison. The tyrosine and leucine residues in the double YXXL/I motif have been boxed. Dashes represent conserved amino acids. Asterisks indicate the positions of the transmembrane lysine and arginine residues.

Fig. 2. Transfection of mutated TCRβ cDNA results in low TCR/CD3 expression. The TCRβ-negative Jurkat clone 31-13 was transfected with either wild type (WT) or mutated (MUT) TCRβ cDNA. The expression of the TCR/CD3 complex on the cell surface in each clone was analyzed by indirect flow cytofluorimetry with CD3 mAb UCHT1. The y axis represents the percentage of TCR/CD3-positive cells in each clone. Clones with less than 5% positive cells were omitted.

CD3ζ Involvement in TCR-CD3-mediated Apoptosis

Fig. 3. TCR-CD3 complex expression in WT and MUT clones. A, large amounts of immature TCR-CD3 complexes are expressed in MUT C2 clone. [35S]Methionine-labeled WT B7 and MUT C2 clones were immunoprecipitated with CD3ε mAb SP34 or TCRβ mAb JOVI-3. The immunoprecipitates were subjected to two-dimensional electrophoresis, first under nonreducing conditions, followed by reducing conditions. The positions of the most relevant proteins are indicated. αζ and βζ, mature forms; αi and βi, immature forms. B, mutated TCRβ chain mRNA is overexpressed in MUT C2 cells. Total RNA from WT B7 or MUT C2 cells was transferred to nylon membranes after denaturing electrophoresis and probed with Vβ3, TCRα, and CD3ζ chains, and ribosomal 28 S specific probes. Vβ3 messenger RNA shows five times more expression in MUT C2 than in WT B7 cells.

1) shows a high degree of homology, therefore suggesting that this domain plays a role other than anchoring the chain in the cell membrane. The extent of residue conservation in this domain is much lower when TCRβ is compared with TCRγ, its analogue chain in γδ T cells. Interestingly, an ITAM-like motif, as defined by the sequence (YXXLXXXXXLYXXI), is present only in the transmembrane domains of TCRβ and TCRγ, whereas the tyrosine residues of the motif are absent in TCRα and TCRβ. It is significant, however, that in TCRα both tyrosine residues are conservatively substituted by phenylalanine residues.

To investigate the putative role of the TCRβ ITAM-like motif, its C-terminal tyrosine residue (named Tyr-TM11) was mutated to leucine using a PCR-based mutagenesis protocol. WT and MUT versions of TCRβ HA1.7 cDNA were transfected into the 31–13 TCRβ minus Jurkat mutant cells previously described (15). After selection in the presence of G-418, WT and MUT TCRβ transfectants were analyzed by flow cytofluorim-
were not detected on the surface of Jurkat, 31–13, and trans-
pressed in all clones, and, as expected, HLA class II molecules
reconstitute the TCR
but also with the anti-V
the complex. All six clones were positive not only with UCHT1
experiments with three WT clones expressing similar levels of
expressing high levels of TCR
possible functional implication of Tyr-TM11, three MUT clones
mutated TCR
expression of the TCR
clones remain in the 10–30% region. The reduced surface ex-
lysed, and immunoprecipitated with either CD3
mAb to determine whether transfection of
antibody N39.

lysis using UCHT1 mAb to determine whether transfection of
TCRβ chain cDNA had reconstituted the expression of the
TCR-CD3 complex. As shown in Fig. 2, WT clones expressed
surface TCR/CD3, with a mean percentage of positive cells of
58%. On the other hand, MUT clones expressed, on average,
only 25% positive cells. In this regard, Fig. 2 shows a clustering
of the WT clones in the 60–80% positive region while MUT
clones remain in the 10–30% region. The reduced surface ex-
pression of the TCR-CD3 complex in clones containing the
mutated TCRβ suggests that this protein does not efficiently
reconstitute the TCR-CD3 complex. To further analyze the
possible functional implication of Tyr-TM11, three MUT clones
expressing high levels of TCR-CD3 were used in comparative
experiments with three WT clones expressing similar levels of
the complex. All six clones were positive not only with UCHT1
but also with the anti-Vβ3 mAb JOVI-3, which recognizes the
variable region of the transfected TCRβ HA1.7. On the con-
trary, none of the clones were stained with a specific Vβ8
antibody, showing that TCR-CD3 expression in these clones
had not resulted from a reversion and reexpression of endoge-
nous Jurkat's TCRβ. Other T cell markers were equally ex-
pressed in all clones, and, as expected, HLA class II molecules
were not detected on the surface of Jurkat, 31–13, and trans-
ferred cells (data not shown).

Mutation of Tyr-TM11 from TCRβ Results in an Impaired
Association of CD3ζ—To investigate the causes of the possible
assembly defect, WTB7 and MUTC2 cells were metabolically
labeled with [35S]methionine for 4 h, and their lysates were
immunoprecipitated with CD3ε and TCRβ-specific mAbs. As
shown in Fig. 3A, JOVI-3 immunoprecipitated a TCRα/β heter-
edromer from WTB7 cells that had the mobility of the mature
Golgi processed form. However, in MUTC2 cells, JOVI-3
mainly immunoprecipitated a TCRα/β heterodimer that
migrated with the characteristic mobility of the immature
heterodimer located in the endoplasmic reticulum (ER) (19).
Nevertheless, some mature TCRα/β heterodimer was also
detected in MUTC2 cells, accounting for the surface staining of
these cells. In addition to the mature and immature heterodimer,
large amounts of unassociated TCRβ chain were detected in
MUTC2 cells, accounting for the surface staining of these
cells. In addition to the mature and immature heterodimer,
large amounts of unassociated TCRβ chain were detected in
MUTC2 cells, accounting for the surface staining of these
cells.
some CD3ζ homodimer was detected in metabolic labelings of WTB7 cells (Fig. 3A), none was detectable in MUTC2. However, due to the poor labeling of CD3ζ, we could not rule out the existence of this subunit. To further explore this possibility, WTB7 and MUTC2 cells were surface iodinated by the lactoperoxidase method prior to exposing the cells to sulfo-succinimidyl-3-(4-hydroxyphenyl)-propionate, previously shown to enhance the detection of CD3ζ (16). After iodination, the cell lysates were immunoprecipitated with CD3e- and CD3ζ-specific mAbs. As shown in Fig. 4, immunoprecipitation with CD3ζ antibody N39 from the WTB7 lysate resulted in the coprecipitation of CD3ζ, -δ, and -ε as well as the TCRα/β heterodimer. On the other hand, although immunoprecipitation with anti-CD3ζ from MUTC2 revealed similar, if not higher, amounts of CD3ζ to those found in WTB7, the amounts of coprecipitated CD3ζ, -δ, and -ε chains and TCRα/β were greatly diminished. Conversely, the immunoprecipitation with a CD3e mAb showed that the levels of CD3ζ, -δ, -ε, and TCRα/β were similar in WTB7 and MUTC2 cells, while the levels of complex-associated CD3ζ were much lower in MUTC2 than in the WTB7 clone (Fig. 4). These experiments suggest that, on the MUTC2 cell surface, CD3ζ is loosely associated in the TCR-CD3 complex and mostly present as an independent molecule.

**Mutation of Tyr-TM11 Abrogates TCR-CD3-induced Programmed Cell Death but Not Other Activation Events**—To test the effect of Tyr-TM11 mutation on TCR-CD3-mediated signaling, MUT and WT clones were stimulated with PMA and different concentrations of soluble UCHT1 mAb; after 24 h, IL-2 production was measured in a CTLL2 proliferation assay. Although some intrinsic variability was found among the different clones, there was not a clear effect of the mutation in the ability of the TCR-CD3 complex to promote IL-2 secretion (Fig. 5A). Similar results were obtained when soluble JOVI-3 mAb; or plastic-bound UCHT1 and JOVI-3 mAbs were used (data not shown). As expected, the parental TCR-CD3-negative Jurkat mutant 31–13 was unresponsive. However, a very clear effect of Tyr-TM11 mutation on cell viability was observed. Consistently, stimulation with plastic-bound UCHT1 or JOVI-3 mAbs resulted in significant losses of cell viability, in WT clones but not in MUT ones, as measured by trypan blue dye exclusion (Fig. 5B).

Upon stimulation with different ligands of the TCR-CD3 complex, a clear dose-response effect on cell viability was observed. That this phenomena is a dose-dependent effect is shown in Fig. 6. Clearly, clone MUTC2 was much less sensitive to stimuli-induced cell death than clone WTB7. Interestingly, soluble Staphylococcus aureus enterotoxin B (SEB), which has been shown to be a ligand for human TCR Vβ3 (21) even in the absence of class II molecules (17, 22), was able to induce a similar effect (Fig. 6C). The mutation of Tyr-TM11 in TCRβ, therefore, abrogated TCR-CD3-induced cell death but did not affect IL-2 secretion.

T cells have been described to undergo apoptosis when stimulated with TCR-CD3 ligands (23–25). To determine whether apoptosis is the mechanism underlying cell death in our cell system, WTB7 and MUTC2 cells were stimulated with various concentrations of soluble SEB, and DNA degradation was measured by propidium iodide staining. The percentage of cells with sub-G1 amounts of DNA is indicated in each panel.

**Fig. 6. Dose-response effect of TCR/CD3 stimulation on cell viability.** WTB7 (open squares) and MUTC2 (filled squares) clones were incubated in plastic wells coated with the indicated concentrations of UCHT1 (A), JOVI-3 (B), mAbs or, alternatively, with soluble SEB (C). Cell viability was measured after 48 h by trypan blue dye exclusion.
MUTC2 clones were stimulated for 4 h at 37°C with JOVI-3 mAb (10 μg/ml) plastic-bound Vβ3 mAb JOVI-3. The expression of CD25 and CD69 was analyzed by flow cytometry after TCR/CD3 stimulation. B, WTG7 and MUTC2 clones were stimulated for 4 h at 37°C with JOVI-3 mAb (10 μg/ml). TCR/CD3 expression was analyzed by flow cytometry after staining the cells with a FITC-conjugated anti-CD3 mAb. Broken lines represent negative controls of staining as determined by using irrelevant FITC-conjugated antibodies.

in WT and MUT cells with similar efficiencies. Taken together, all previous data indicate that mutation of Tyr-TM11 in TCRβ results in a specific inhibition of TCR-CD3-induced apoptosis but has no effect in IL-2 production, CD25 and CD69 expression, and TCR-CD3 complex down-regulation.

TCR-CD3 Stimulation of MUT Cells Results in Reduced Induction of CD95 Ligand Expression Although the Cells Remain Fully Sensitive to CD95-induced Apoptosis—The pathways for activation of apoptosis are multiple and distinct in different cell types. Recently, it has been described that in T cells, TCR-CD3 cross-linking induces both CD95 (also known as APO-1 and Fas) and CD95 ligand (CD95-L) expression and that TCR-CD3-induced programmed cell death is mediated by engagement of CD95 by its ligand (23–25). In Jurkat cells, CD95 expression is constitutive, and TCR-CD3-induced cell death is exclusively mediated by the induction of CD95-L expression (23). We decided to test whether the different sensitivity to TCR-CD3-induced apoptosis in WT and MUT cells was dependent on differences in the CD95 pathway. As shown in Fig. 9A, WTG7 and MUTC2 clones expressed constitutively comparable levels of CD95 on the cell surface. In addition, the levels of expression of CD95 remained stable in both cell types upon TCR/CD3 stimulation with specific mAbs (data not shown). The signaling pathway through CD95 was functional because similar levels of DNA degradation could be induced when either WT or MUT cells were activated via CD95 with a specific anti-CD95 mAb, CH-11, which is able to induce apoptosis (Fig. 9B). Furthermore, stimulation by a TCR-independent mechanism, phorbol ester plus calcium ionophore (Fig. 9C), confirmed that MUT cells are not intrinsically defective in the apoptotic machinery.

On the other hand, soluble DX2, an anti-CD95 mAb previously shown to block CD95/CD95-L interaction, was able to prevent SEB (Fig. 9D) and UCHT1- or JOVI-3 (not shown) induced cell death in WTG7 cells. These results suggested that, as previously shown in Jurkat (23), apoptosis in WT cells was mediated via CD95/CD95-L interaction. Therefore, it was compelling to test whether induction of CD95-L expression was produced upon TCR-CD3 triggering. As shown in Fig. 9E, stimulation with SEB resulted in expression of CD95-L mRNA in WTG7, whereas in MUTC2 cells such expression was highly reduced. The same results were obtained when other WT and MUT clones were tested (data not shown). However, stimulation with phorbol ester plus ionophore resulted in similar induction of CD95-L in WT and MUT clones (data not shown) suggesting, again, that signaling to apoptosis through the CD95/CD95-L pathway takes place in MUT cells when a stimulus that bypasses TCR-CD3 is used.

These results suggest that mutation of Tyr-TM11 in TCRβ results in defective CD95-L induction in Jurkat transfectants and that this is the basic mechanism underlying the resistance to TCR-CD3-induced apoptosis of these cells.

**DISCUSSION**

We have investigated the effects of replacing a highly conserved tyrosine residue in the transmembrane region of TCRβ chains, on assembly and signal transduction. Interestingly, this residue lies in a sequence that resembles consensus ITAM motifs previously described in the cytoplasmic tails of multi-subunit receptors including those of the CD3γ, -δ, -ε, and -ζ chains of the TCR-CD3 complex (13). Two major effects were observed upon transfection of the MUT TCRβ chain cDNA in Jurkat TCRβ negative cells. First, an impaired intracellular association of CD3ζ was observed that apparently resulted in the surface expression of a TCR-CD3 complex loosely associated with the CD3ζ chain. Second, and more interesting, a specific inhibition of apoptosis was induced upon TCR-CD3 stimulation. As all other measured T cell activation events were not affected by the mutation, our results suggest the existence of an independent intracellular signaling pathway for apoptosis in which CD3-ζ seems to be involved.

The conspicuous location of the ITAM-like motif makes it difficult to explain how Tyr-TM11 could play a role in transmembrane signaling. Recently, however, a conserved antigen receptor transmembrane (CART) motif has been described (26), which shares homology with the ITAM-like motif we describe here. Based on theoretical grounds, the authors suggested that several amino acids, including Tyr-TM11, could be important in antigen receptor function. The mutational studies performed on the transmembrane domain of surface IgM implicating polar...
sequences in intersubunit interactions and cell activation support this idea (27–29). Curiously, it has been recently reported that the selective point mutation of a similar tyrosine residue included in an intracytoplasmic ITAM motif completely abrogated the ability of this motif to mediate cell death signals (30). However, the expected behavior of the β chain transmembrane domain as a target for a protein tyrosine kinase has not been reported, and our attempts to demonstrate such potential susceptibility have been unsuccessful. If this failure reflects either technical problems or natural incapacity of the TCR β chain to be phosphorylated will require additional and more specific experiments.

Although the mutation of Tyr-TM11 could directly affect the association with a not yet defined protein that would mediate triggering of apoptosis, a more plausible explanation for the presented data is that the impaired association of CD3ζ in the complex is the cause of defective apoptosis. Hence, this subunit would be directly involved in the activation of the cell death program. This idea is reinforced by the data of Vignaux et al. (31) showing that cross-linking of a Tac/ζ chimera, containing the cytoplasmic tail of CD3ζ, induces apoptosis in a murine T cell hybridoma. Furthermore, it has recently been shown that in vitro cross-linking of Tac/ζ induces double-positive thymocyte cell death from Tac/ζ transgenic RAG2−/− mice (32). The same effect was observed by cross-linking of Tac/ε chimera in Tac/ε transgenic RAG2−/− mice. If these data reflect either a redundant role for the ε and ζ chains in delivering qualitatively similar signals for inducing apoptosis or the result of nonphysiological stimuli remains controversial. On this line of evidence it has been reported that the role of individual ITAM-containing chains may have qualitatively different functions that can control the TCR-CD3 triggering under different physiological situations depending upon the activation status of the cell (33).

It has been recently described that the route leading to apoptosis triggering by the TCR-CD3 complex in murine hybridomas and in Jurkat cells is mediated by the CD95 pathway. While in Jurkat cells CD95 is constitutively expressed, CD95-L is rapidly up-regulated, after SEB or anti-CD3 stimulation, leading to induction of cell death (23–25, 34). However, we have shown that while apoptosis triggering by direct stimulation of CD95 is not affected by the mutation, the induction of CD95-L upon TCR/CD3 triggering is inhibited. Thus, the CD95-L induction seems to be the basic phenomenon affected by Tyr-TM11 mutation and probably by the impaired association of CD3ζ. This idea is supported by the fact that cross-linking of a Tac/ζ chimera was capable of inducing CD95-L expression (31).

On the other hand, the mutation of Tyr-TM11 of TCR β did not affect other activation events, implying that a CD3ζ minus TCRζCD3 complex may be sufficient to trigger other activation programs. In this regard, a CD3ζ minus module has previously

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**Fig. 9. Failure in CD95 ligand induction is responsible for the resistance to SEB or anti-TCR/CD3 mAb-induced programmed cell death in MUT clones.**

A. Expression of CD95 in nonstimulated WTB7 and MUTC2 clones. Cells were stained with 4 μg/ml anti-CD95 mAb DX2 and analyzed by flow cytometry. Dashed lines represent the fluorescence intensity for 97% of mock stained cells. B. DNA degradation. WTB7 and MUTC2 cells were treated with 500 ng/ml anti-CD95 mAb CH11. After 48 h, DNA degradation was measured by propidium iodide staining. The percentage of cells with sub-G₀ amounts of DNA is indicated in each panel. C. DNA degradation induced upon addition of phorbol ester plus calcium ionophore. WTB7 and MUTC2 cells were incubated with 1 μM calcium ionophore A23187 plus 15 ng/ml PMA. After 24 h, DNA degradation was measured as in B. D. Inhibition of SEB-induced cell death with anti-CD95 mAb DX2. DNA degradation was measured by propidium iodide staining in WTB7 and MUTC2 cells treated for 48 h with 10 μg/ml SEB in the presence of 500 ng/ml DX2. The percentage of cells with sub-G₀ amounts of DNA is indicated in each panel. E. Differential expression of Fas-L (CD95-L) in WT and MUT clones. Total RNAs were extracted from WTB7 and MUTC2 cells treated for 48 h with 10 μg/ml SEB for the indicated times. Blots were hybridized with a CD95-L cDNA fragment and rehybridized with a 28S ribosomal probe as loading control.
been shown to be sufficient to induce IL-2 secretion in a murine hybridoma (35). Also consistent with these data, TCR complexes containing a CD3ζ chain deprived of all ITAM motifs have been shown to promote normal T cell maturation in transgenic mice (36). Moreover, double-positive thymocytes from these mice are able to transduce signals resulting in up-regulation of CD69 surface expression, up-regulation of CD5 mRNA, and down-regulation of RAG mRNAs.

The three ITAM motifs of CD3ζ and the one of CD3ε have been shown to bind with different affinities to the SH2 domains of proteins involved in the activation pathway, such as ZAP-70, Shc, and phosphatidylinositol 3-kinase (12, 37, 38). It is, therefore, reasonable to assume that the role of CD3ζ in apoptosis could take place through the specific recruitment, by any of its ITAM motifs, of signal transducing proteins needed for apoptosis triggering but not, for instance, for IL-2 secretion. Alternatively, apoptosis triggering may require a signal threshold that would not be reached in the absence of the six ITAM motifs included in the CD3ζ homodimer. Therefore, the lack of associated CD3ζ may have a quantitative rather than a qualitative effect. Further experiments are required to distinguish between both possibilities.

The data presented in this study are consistent with the idea that the association of CD3ζ is the last step in the assembly of the TCR-CD3 complex because it requires the previous association of CD3γ, -β, and -ε with TCRα and TCRβ. Thus, transient transfection studies performed in COS cells indicated that CD3ζ does not interact directly with TCR or CD3 chains alone, but it requires the previous association of both TCRα and -β to the other CD3 chains in order to provide adequate conformation for binding of CD3ζ (8, 20).

It has been shown that the electrostatic interactions between basic residues in the transmembrane region of TCRα (lysine and arginine), TCRβ (lysine), and single acidic residues in the transmembrane domains of each of the different CD3 chains play an important role in assembly (7). The tyrosine residue mutated in TCRβ is in position +4 of the lysine residue which, assuming an α-helix conformation for the transmembrane domain, would be placed on the same face of the helix. Thus, lysine and tyrosine could form a hydrophilic surface in the transmembrane domain of TCRβ that mediates assembly with the CD3 subunits. Nevertheless, while the basic residues of TCRβ and -α chains appear to be involved in the association with CD3γ, -β, -ε, and -γ mutant Tyr-TM11 seems to be specifically involved in assembly of the CD3ζ chain. Interestingly, mutation of two contiguous hydrophilic amino acids, including a tyrosine, located in the transmembrane domain of the μ chain of the B cell receptor resulted in loss of association with Igα and Igβ chains (29), pointing to a general role of transmembrane tyrosine residues in multisubunit receptor assembly and expression. The effect of Tyr-TM11 mutation on CD3ζ association could be due to a direct interaction between this residue and specific amino acids of CD3ζ, or alternatively, to steric or allosteric effects on other CD3 chains.

Most TCRβ MUT clones expressed lower levels of TCR-CD3 complexes on the cell surface than WT ones. This effect was likely due to a hampered exit of TCR-CD3 complexes from the ER. The inefficient association of CD3ζ chain to the TCRβζ-CD3γ-ε core in the ER probably avoids the transport of complete complexes to the cell surface (9, 10), and only in those clones where mutated TCRβ is overexpressed would the defective association of CD3ζ be compensated. Interestingly, CD3ζ was only partially associated to the remaining TCR-CD3 complex on the cell surface of the MUTC2 clone expressing high levels of the complex, suggesting the existence of an association/dissociation equilibrium both inside the cell and on the plasma membrane. The association of CD3ζ would be necessary for the TCR-CD3 complex to leave the ER but once by-passed the ER retention checkpoint could CD3ζ again dissociate. Our results suggest, therefore, that the composition of the TCR-CD3 complex could fluctuate among several forms at equilibrium. Non-TCR-associated CD3ζ could either remain as a free chain on the cell surface or become associated to other proteins such as the transferrin receptor (16). Interestingly, it has been described that tumor infiltrating T lymphocytes show a marked alteration in the structure of their TCR-CD3 complexes, namely the absence of ζ chain (39–41).

The structural and/or functional significance of the ITAM-like motif located in the transmembrane domains of TCRβ and TCRγ chains is unknown. Our data point out that the C-terminal tyrosine residue of such a motif is implicated in the association with CD3ζ and in the TCR-CD3-mediated signaling, probably via CD3ζ, that leads to apoptosis. The putative role of other amino acids of the motif in assembly and signal transmission through the TCR-CD3 complex will require further investigation.

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Note Added in Proof—While this manuscript was under revision, a paper by Combadière et al. (Combadière, B., Freedman, M., Chen, L., Shover, W., Love, P., and Lenardo, M. J. (1996) J. Exp. Med. 183, 2109–2117) was published, which by other approaches confirms the implication of CD3ζ in TCR signaling to apoptosis.

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CD3ζ Involvement in TCR-CD3-mediated Apoptosis

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