Qualitative and Quantitative Contributions of the T Cell Receptor ζ Chain To Mature T Cell Apoptosis

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Summary

Engagement of the T cell receptor (TCR) of mature T lymphocytes can lead either to activation/proliferation responses or programmed cell death. To understand the molecular regulation of these two fundamentally different outcomes of TCR signaling, we investigated the participation of various components of the TCR–CD3 complex. We found that the TCR-ζ chain, while not absolutely required, was especially effective at promoting mature T cell apoptosis compared with the CD3ε, γ, or δ chains. We also carried out mutagenesis to address the role of the immunoreceptor tyrosine-based activation motifs (ITAMs) that are the principal signaling components found three times in the TCR-ζ chain and once in each of the CD3ε, γ, or δ chains. We found that the ability of the TCR-ζ chain to promote apoptosis results both from a quantitative effect of the presence of multiple ITAMs as well as qualitatively different contributions made by individual ITAMs. Apoptosis induced by single chain chimeras revealed that the first ζ ITAM stimulated greater apoptosis than the third ζ ITAM, and the second ζ ITAM was unable to trigger apoptosis. Because microheterogeneity in the amino acid sequence of the various ITAM motifs found in the TCR-ζ and CD3 chains predicts interactions with distinct *src*-homology-2-domain signaling proteins, our results suggest the possibility that individual ITAM motifs might play unique roles in TCR responses by engaging specific signaling pathways.

Programmed cell death (PCD)[1] or apoptosis is a regulated process by which multicellular organisms eliminate unneeded or harmful cells (1, 2). In the immune system apoptosis of lymphocytes that is triggered by antigen receptors, i.e., Ig on B lymphocytes and the TCR on T lymphocytes, is crucial for the establishment of the lymphocyte repertoire and the clonal regulation of immune responses (3–5). Apoptosis caused by TCR signals can occur at two different points in the life of a T cell. Apoptotic death occurs first during differentiation in the thymus where strong TCR engagement may lead to negative selection, which is an important means of establishing self-tolerance through the elimination of cells with autoreactive TCRs (6–8). TCR-induced apoptosis may later befall a fully mature T cell if strong TCR reengagement occurs when the cell is activated and proliferating (9–11). Mature T cell death plays a critical role in peripheral immune homeostasis and tolerance (5). Evidence would suggest that the molecular mechanisms of TCR-induced apoptosis in developing thymocytes are not the same as those in mature, peripheral T cells (12).

Involvement of the TCR in both the activation and death of mature T cells raises the interesting question of how these different outcomes are regulated. The TCR–CD3 complex is an oligomeric structure, composed of multiple chains (TCR-α/β or -γ/δ associated with CD3δ, γ, ε, and ζ or η). Antigen recognition is accomplished by the polymorphic TCR-α/β (or -γ/δ) heterodimers. The invariant CD3 proteins (γ/ε, δ/ε dimers) and the ζ/η proteins (ζ/ζ, ζ/η dimers) promote efficient cell surface expression and transduce TCR signals (13, 14). Signaling requires a conserved 18–amino acid sequence, termed the immunoreceptor tyrosine-based activation motif (ITAM), which is found three times in the TCR-ζ chain and once in each of the CD3 subunits (γ, δ, and ε) (15–17). Each ITAM contains a pair of tyrosine-X-X leucine/isoleucine (Y-X-X-L/I) motifs, that are separated by 10/11 amino acids (17). The tyrosine residues in each ITAM are rapidly phosphorylated after TCR ligation and serve as docking

[1] *Abbreviations used in this paper:* ITAM, immunoreceptor tyrosine-based activation motif; PCC, pigeon cytochrome C; PCD, programmed cell death; PI, propidium iodide; SH2, *src*-homology-2.

Drs. Combadière and Freedman contributed equally to this paper.

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sites for signaling proteins that can bind to the phosphotyrosine residues by *src*-homology-2 (SH2) domains (18–24). Deletions of the ITAM or mutations of the tyrosines within the Y-X-X-I/L motifs cause a loss of function indicating their vital role in TCR signaling (25–27). There is heterogeneity in the amino acids adjacent to the phosphotyrosines in the various ITAMs found in the ζ and CD3 chains (see Table 1). Experiments by Songyang et al. (22, 23) show that these amino acids determine the specificity of binding of SH2 domains, suggesting that individual TCR ITAMs could couple to distinct SH2 domain signaling proteins (22, 23). We therefore explored the idea that distinct chains and/or ITAMs of the oligomeric TCR–CD3 complex could have an especially important role in initiating apoptosis.

Studies of chimeras between the extracellular domain of non-TCR proteins, such as IL-2Rα (Tac) or CD8, and the intracytoplasmic portions of the TCR–CD3 chains have revealed that the cytoplasmic tail of the TCR-ζ or CD3ε can couple external signals to both proximal (induction of tyrosine phosphoproteins, Ca^{2+} and inositol phosphates) and distal (IL-2 secretion, CD69 expression) signaling functions (26–31). Chimeric molecules containing either the CD3ε or TCR-ζ cytoplasmic domains are also capable of mediating thymocyte maturation, proliferation, and selection (32). Therefore, the view has emerged that there are two independent signaling modules with overlapping functions for activation: one mediated by the TCR-ζ chain and one mediated by the CD3 chains, especially CD3ε (33, 34). Recent analyses of cytotoxic T cells have shown that the TCR-ζ cytoplasmic domain can upregulate Fas-dependent cytolysis of target cells, but the CD3ε chain has not been examined in this regard (35). To what extent there is redundancy in signal pathways for autoregulatory mature T cell apoptosis has not been investigated. We therefore tested the role of the TCR-ζ or CD3ε, γ, or δ chains and their ITAM motifs in single chain chimeras and in the context of a complete receptor for the ability to induce mature T cell apoptosis.

Materials and Methods

Constructs. Chimeric proteins containing the external and transmembrane portions of the Tac protein associated with cytoplasmic portions of either ζ, γ, δ, or ε were prepared as previously described (29, 36, 37). Briefly, DNA segments encoding the relevant portions of the CD3 chains were obtained by PCR, cloned into pCDL SRα (36, 37), and verified by DNA sequence analysis. The sequences of DNA primers used were the following: 5′ CCA TAT TTA CAA CAG ATC TCC AGG 3′ present in Tac transmembrane domain and 5′ GTC CAA ACT ACT CAT 3′ present in PCDL SRα vector. DNA segments encoding for mutated tyrosines of the ζ cytoplasmic chain into phenylalanine were obtained by PCR using two sets of overlapping primers containing a point mutation as follows: first ITAM of the ζ chain (Y72, Y83 mutated into F), 3′ primer [5′ GAG CTC AAT CTA GGG CGA AGA GAG GAA TTT GAC GTC TTG], 5′ primer [5′ CTT CGC CCT AGA TTG AGC TCA TTG AAG AGC TGG TTG]; second ITAM of the ζ chain (Y111 and Y123 mutated into F), 3′ primer [5′ GCA GAA AGA CAA GAT GGC AGA AGC CTT CAG TGA GAT C], 5′ primer [5′ CTT CTG CCA TCT TGT CTT TCT GCA GTG CAT TGA ATA CGC CTT]; and third ITAM of the ζ chain (Y143, Y153 mutated into F), 3′ primer [5′ CTC AGC ACT GCC ACC AAG GAC ACC TTT GAT GCC CTG], 5′ primer [5′ GTG TCC TTG GTG GCA GTG CTG AGA CCC TGG AAA AGG CCA TC]. Z1ε, Z2ε, Z3ε, GET, and DET are five chimeric molecules containing Tac extracellular and transmembrane domains and the full CD3ε cytoplasmic domain in which the ε ITAM has been replaced by either the TCR-ζ ITAM 1, TCR-ζ ITAM 2, TCR-ζ ITAM 3, CD3γ, or CD3δ ITAM, respectively, by using two sets of overlapping primers as follows: Z1ε 3′ primer [5′-GGG CGA AGA GAG GAA TAT GAC GTC TTG AAT CAG AGA GCA G], 5′ primer [5′-CCT CTC TTC GCC CTA GAT TGA GCT CAT TGT AGT CTG GGG TTG G]; Z2ε 3′ primer [5′-GAC AAG ATG GCA GAA GCC TAC AGT GAG ATC AAT CAG AGA GCA G], 5′ primer [5′-CTG CCA TCT TGT CTT TCT GCA GTG CAT TGT AGT CTG GGT TGG G]; Z3ε 3′ primer [5′-GTG CCA GTG CTG AGA CCC TGA TAG TCT GGG], 5′ primer [5]-CAG CAC TGC CAC CCA GGA CAC CTA TGA TGC CTG AAT CAG AG]; GET 3′ primer [5′-CCG GGA ATA TGA CCA GTA CAG CCA TCT CAA TCA GAG AGC AG], 5′ primer [5′-TCA TAT TCC CGG TCC TTG AGG GGC TGG TAG TCT GGG TTG GG] ; and DET 3′ primer [5′-CGT GAA GAT ACC CAG TAC AGC CGT CTT AAT CAG AGA GCA], 5′ primers [5′-GGG TAT CTT CAC GAT CTC GAA GAG GCT GAT AGT CTG GGT TGG]. The TCR-ζ, CD3ε, CD3γ, and CD3δ ITAM amino acid sequences used are shown in Table 1. CD3 chimeras were necessary because Tac fusion proteins containing the full cytoplasmic portions of the CD3γ or CD3δ chains are unstable and have very poor surface expression (29). The single letter amino acid code is: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Cell Culture. Tac clones were cells expressing chimeric proteins comprised of the Tac extracellular and transmembrane fused to the intracytoplasmic portion of the TCR-ζ chain (termed TT-ζ) or the CD3ε chain (termed TT-ε). The clones TT-ζ4 and TT-ε5 were a gift from Dr. R.D. Klausner (National Cancer Institute, Bethesda, MD). For new clones, constructs were stably expressed in BW5147($\alpha^-\beta^-$) (BW), a variant of the mouse BW cell line that synthesizes no α, β, δ, or γ chains (29). BW cells were transfected by electroporation with 18 μg of plasmid linearized by HindIII and 2 μg of pGEMneo cut by EcoRI per 10^7 cells. A voltage of 350 V and a capacitance of 1,300 μF were applied by an electroporation unit (BTX, San Diego, CA). Stable transfectants were selected with 1.5 mg/ml of geneticin (G148) (GIBCO BRL, Gaithersburg, MD). Clones were obtained by limiting dilution according to the Poisson distribution. Intensity of Tac expression on the cell surface was determined by flow cytometry.

Table 1. *Sequences of Mouse TCR-CD3 ITAM*

TCR-ζ1	Q L **Y** N E **L** N L G R R E - **Y** D V **L**
TCR-ζ2	G V **Y** N A **L** Q K D M A E A **Y** S E **I**
TCR-ζ3	G L **Y** Q G **L** S T A T K D T **Y** D A **L**
CD3γ	Q L **Y** Q P **L** K D R E Y D Q **Y** S H **L**
CD3ε	P D **Y** E P **I** R K G Q R D L **Y** S G **L**
CD3δ	Q L **Y** Q P **L** R D R E D T Q **Y** S R **L**

For transgenic mouse studies, lymph node T cells were obtained from $\zeta^{-/-}$ mice with a full-length ζ transgene ($\zeta^{-/-}$:Tgζ) or $\zeta^{-/-}$ mice with a transgene expressing a truncated ζ molecule lacking the cytoplasmic domain (amino acids 67-150) ($\zeta^{-/-}$: Tg$\zeta\Delta$67-150 (38, 39). The cells were stimulated first for 48 h with 30 ng/ml of phorbol dibutyrate (PDB; Sigma Chemical Co., St. Louis, MO) and 1 μm/ml of ionomycin (Calbiochem-Novabiochem Corp., San Diego, CA), washed, and then stimulated with 50 IU/ml of human recombinant IL-2 (Proleukin; Cetus Corp., Emeryville, CA) for an additional 48 h as previously described (9, 40). Proliferation assays, culture supernatant collection, and apoptosis induction assays were then performed.

Apoptosis Induction Assay. Cells (5×10^4) were cultured in triplicate in 96-well flat bottom plates in a final volume of 200 μl. Anti-Tac (gift from Dr. Thomas Waldmann, National Cancer Institute, Bethesda, MD) or anti-CD3 (145-2C11) (41) stimulation was performed using plates precoated at 37°C for 16 h with various concentrations of mAb dissolved in 100 μl of PBS. It is noteworthy that the anti-Tac mAb used in these studies (42) appears to be a weaker agonist than the commercially available mAb (33B3.1) used in previous studies of Tac-TCR subunit chimeras (29). After 24 h of incubation of the cells at 37°C, 100 μl supernatant was harvested for IL-2 assay and the number of viable cells was measured cytofluorometrically (43). Briefly, cells were removed from culture wells by vigorous pipetting, washed once in PBS, and resuspended in a constant volume of FACS® buffer (PBS 1%, BSA 0.05%, sodium azide) and propidium iodide (PI; 20 μg/ml). Each sample was prepared in duplicate. Samples were analyzed on a FACScan® II instrument (Becton Dickinson & Co., Mountainview, CA) for a constant period of time (instead of collecting a constant number of events, without live gating). The number of viable cells per period of collection was gated by forward scatter profile and PI exclusion. The percentage of loss was calculated as $100 \times [1-$(number of viable cells receiving Ab or TCR stimulation/number of viable cells without any treatment)]. For antigen stimulation experiments, 2B4.11 and a derivative cell line MA $\zeta\Delta$66-157 (gifts from Dr. Bernard Malissen, Inserm-CNRS, Marseille-Luminy, France) were used (33, 34, 44). Samples containing 5×10^4 cells were incubated with irradiated (3,000 rad) H-2E^k splenocytes (5×10^5) from B10.A mice, and various concentrations of pigeon cytochrome C peptide 81-104 (PCC p81-104) in a final volume of 200 μl. After 24 h of incubation at 37°C, 100 μl of supernatant was harvested for the IL-2 assay and the level of T cell death was quantitated as described previously (5, 11, 40, 43). Cell surface expression of the chimeric proteins was detected by staining with an appropriate dilution of PE-conjugated anti-CD25 (PharMingen, San Diego, CA) for 20 min followed by analysis using a FACScan® II analyzer with CellQuest™ software (Becton Dickinson & Co.).

IL-2 Assay. IL-2 was detected by ELISA using Abs purchased from PharMingen, essentially as per manufacturer's instructions. Data are expressed as IL-2 U/ml, calculated by comparison with standard curve determined with recombinant mouse IL-2 (PharMingen).

Analysis of DNA Fragmentation. Fragmentation of the genomic DNA isolated from cells cultured on plate-bound anti-Tac mAb was analyzed by agarose gel electrophoresis as previously described (40).

Results and Discussion

The Tac-ζ Chimeric Protein Can Induce Programmed T Cell Death. We first studied apoptosis induction using two clones, TT-ζ4 and TT-ζ7 (29), that express chimeric proteins containing the extracellular and transmembrane domains from CD25 (Tac) and the cytoplasmic domain of the TCR-ζ chain. Biochemical analyses indicated that these chimeric molecules do not associate with endogenous CD3 subunits and therefore act as physically independent signaling molecules (32). When we cross-linked the TT-ζ chimeric molecule, we detected dramatic induction of apoptosis as indicated by the microscopic appearance of the cells and the electrophoretic pattern of cleaved genomic DNA (Fig. 1, *A* and *B*). The TT-ζ4 and TT-ζ7 clones had a similar surface expression of Tac chimeric molecules (analyzed cytofluorometrically, data not shown) and showed the same dose–response for apoptosis as measured by PI inclusion (Fig. 1 *C*). The BW parental cell line did not die at any dose of anti-Tac tested (Fig. 1 *C*). BW cells were not innately resistant to death because phorbol ester and ionomycin caused the death of BW cells equivalent to the TT-ζ4 and TT-ζ7 clones (Fig. 2 *C*). These initial results confirmed and extended the observations of Vignaux et al. (35) who found that TCR-ζ chain could induce Fas ligand on cytolytic T cells at levels sufficient to kill target cells. We also found that TCR-ζ chain signals induced Fas and Fas-ligand mRNA (data not shown). However, in our model, TCR-ζ signaling was associated with apoptosis of the T cells themselves, similar to autoregulatory T cell apoptosis, which plays an important role in peripheral tolerance (45).

TCR–CD3ζ Subunits Lacking the Cytoplasmic Domain Failed to Efficiently Induce T Cell Apoptosis. We next evaluated the role of the TCR-ζ chain in mature T cell death by analyzing the MA5.8 cell line, which is a mutant derived from the 2B4.11 hybridoma that synthesizes all of the TCR components except the TCR ζ/η proteins and therefore has no surface expression of the TCR (29, 44). TCR surface expression can be restored by expression constructs for the TCR-ζ chain either with or without a cytoplasmic domain (33, 34, 44). We compared a MA5.8 transfectant, MA $\zeta\Delta$66-157, that lacked almost the entire cytoplasmic portion of the TCR-ζ chain, including all three ITAMs (amino acids 66–157), to parental 2B4 cells that expressed a full-length ζ chain. This allowed us to evaluate the importance of TCR-ζ chain signaling in mature T cell death within the context of the oligomeric TCR complex.

After stimulation with immobilized anti-CD3ε mAb, we found that apoptosis was substantially less for the MA5.8 cells lacking the intracytoplasmic domain of ζ (Table 2), compared with 2B4 cells. Similar results were obtained after stimulation of the hybridomas with the physiological PCC peptide ligand (Table 2). Although peptide stimulation caused less overall death than cross-linking Ab, the absence of the TCR-ζ cytoplasmic signaling domain was associated with a 5–10-fold reduction in the level of antigen-induced apoptosis. These observations contrast with previous work showing that IL-2 can be highly induced by a TCR complex that lacks the cytoplasmic portion of the TCR-ζ chain (29, 33, 34). Our results reveal that apoptosis in these T cell hybridomas is most efficiently triggered by TCR complexes that have an intact TCR-ζ cytoplasmic signaling domain.

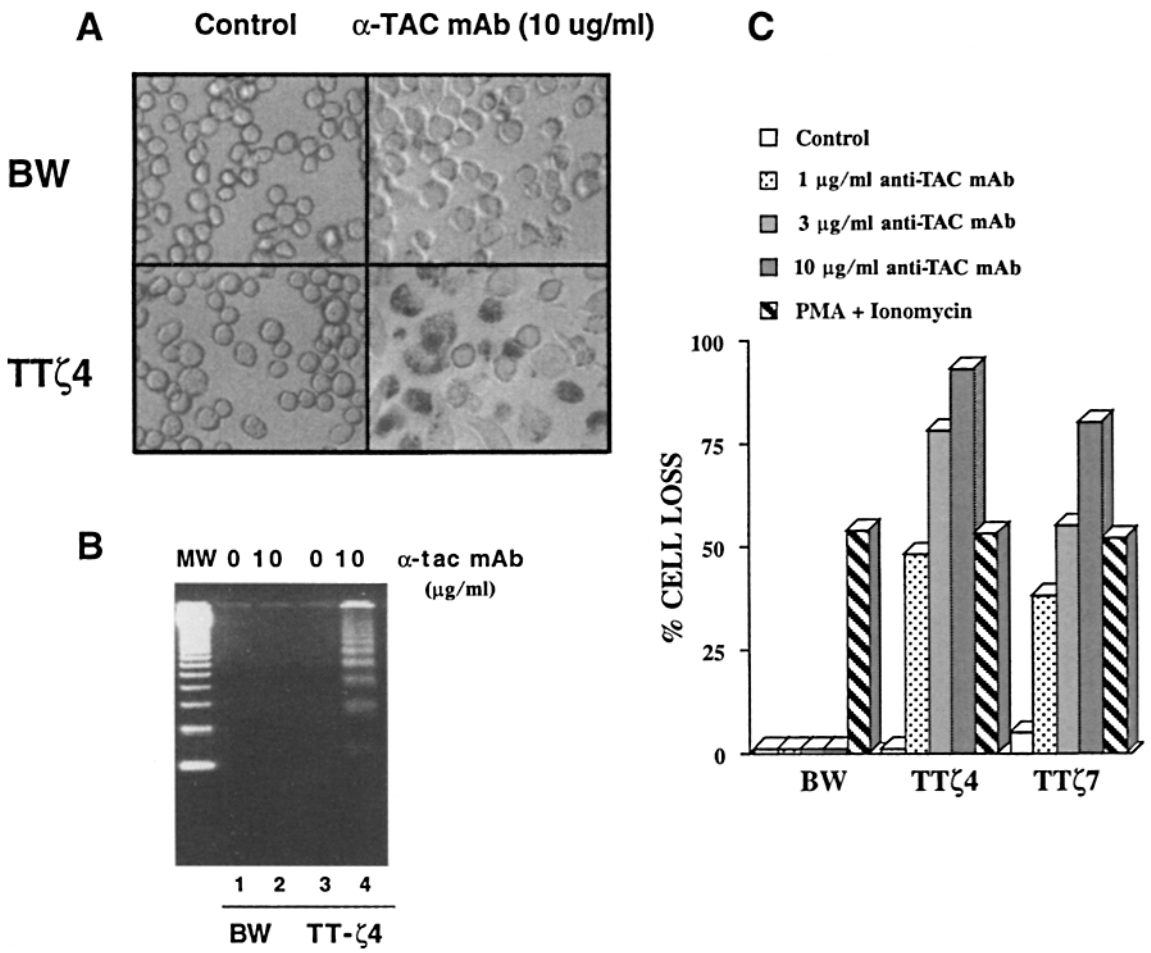

Figure 1. Chimeric proteins bearing the cytoplasmic domain of TCR-ζ chain cause autoregulatory T cell apoptosis. (*A*) Photomicrographs of trypan blue stained–cells after treatment with plate-bound anti-Tac mAb (10 μg/ml) for 24 h. Dead cells are darkly stained and viable cells are unstained. (*B*) Agarose gel electrophoresis of DNA isolated from cells stimulated with anti-Tac mAb. (*MW*) 123-bp ladder DNA molecular weight marker. Samples for lanes *1* and *2* are from BW cells and lanes *3* and *4* are from TT-ζ4 cells. (*C*) TT-ζ4 and TT-ζ7 clones were exposed to 1, 3, or 10 μg/ml of immobilized anti-Tac mAb for 24 h as indicated. The anti-Tac mAb used in these experiments is a weaker agonist than the commercial anti-Tac mAb (33B3.1) used in previous reports (29), and therefore had to be used in greater concentrations. Quantitation of cell loss was determined by flow cytometric analysis using PI as described in Materials and Methods. Control samples were stimulated by PMA (10 μg/ml) and ionomycin (1 μM).

Peripheral Lymph Node T Cells Require the TCR-ζ Cytoplasmic Domain for Efficient TCR-mediated Apoptosis. We next verified the requirement for the ζ signaling domain in the induction of apoptosis of nontransformed lymph node T cells. We used mice that were homozygously deficient for the TCR-ζ gene as well as offspring that had been reconstituted with either full-length TCR-ζ or the ζΔ67-150 derivative which contains no ITAM signalling motifs (38, 39). A previous investigation of these mice found that the cytoplasmic signaling portion of the TCR-ζ modestly improved thymocyte maturation, but was not obligatory (39). We therefore harvested peripheral lymph node T cells from the mouse strains and induced activation and cell cycling with IL-2 to predispose the cells to apoptosis (9). We confirmed that T cells from the reconstituted transgenic lines had comparable surface expression of TCR, CD4, and CD8, whereas the ζ−/− mouse had no surface TCR (38) (Fig. 2 *A* and data not shown). TCR cross-linking at 10 h caused substantial apoptosis in lymph node cells from mice reconstituted with a full-length ζ chain (ζ−/−:Tgζ), but very little in T cells expressing a cytoplasmically deleted ζ chain (ζ−/−:TgζΔ67-150) or no ζ chain at all (ζ−/−) (Fig. 2 *B*). By contrast, we found that peripheral T cells containing the TgζΔ67-150 chain produced just as much IL-2 at various concentrations of TCR cross-linking Ab as T cells expressing the full-length ζ chain (Fig. 2 *C*). These results show that under identical stimulation conditions, the signaling portion of the TCR-ζ chain is dispensable for activation and IL-2 production, but required for apoptosis. The effect of the TCR-ζ chain appeared to involve enhancing the rate of apoptosis because prolonged stimulation of T cells bearing the truncated ζ chain could lead to significant apoptosis (data not shown). However, this effect on the efficiency of signaling appeared to be selective for apoptosis as no difference was observed for activation events such as IL-2 production or IL-2 receptor expression (Fig. 2 *C* and data not shown).

The ζ Cytoplasmic Domain Promotes T Cell Apoptosis More Effectively than the Signaling Domains of the CD3ε Chain. To further assess the function of the TCR-ζ chain in mature T cell apoptosis, we directly compared the signaling

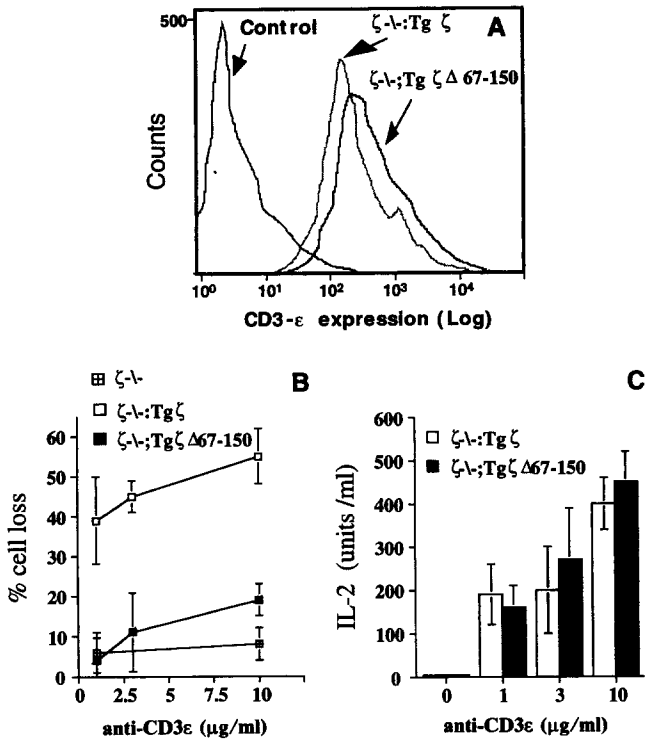

Figure 2. Mature lymph node T lymphocytes require the TCR-ζ cytoplasmic domain for efficient TCR-mediated apoptosis in transgenic mice. Peripheral lymph node cells from ζ−/−, ζ−/−:Tgζ (wild-type TCR-ζ chain) and ζ−/−:TgζΔ67-150 (deletion of TCR-ζ ITAMs from amino acids 67–150) were activated by 30 ng/ml PDB (phorbol 12, 13 dibutyrate) and 1 μg/ml ionomycin, stimulated with 50 IU/ml human IL-2 and then rechallenged with various amounts of plate-bound anti-CD3ε as indicated. (*A*) Flow cytometry histogram of CD3ε expression on activated and cycling lymph node cells from the transgenic mice with a full-length or deleted TCR-ζ chain. Control represents unstained cells. (*B*) Stimulation of activated cells (ζ−/−, *crossed square*, ζ−/−:Tgζ: *open square* and ζ−/−,TgζΔ67-150, *solid square*) was carried out with different concentrations of immobilized anti-CD3ε mAb (145.2C11) for 10 h as indicated. The percent cell loss was calculated by PI exclusion via FACS®. (*C*) IL-2 production for various cell populations was determined by ELISA on supernatant collected at 10 h after anti-CD3 stimulation. The IL-2 units were calculated by comparison with a standard curve using recombinant mouse IL-2.

Table 2. *Anti CD3ε Stimulation Fails to Induce PCD without the TCR-ζ Cytoplasmic Domain*

		Percent cell loss	
		MA ζΔ66-157	Control cell lines (2B4)
		%	
Anti-CD3ε Ab	0.3 μg/ml	0	57
	1 μg/ml	23	74
	3 μg/ml	29	78
	10 μg/ml	30	80
PCC (81-104)	100 μM	3	32
(expt. 1)	10 μM	7	35
(expt. 2)	10 μM	1	ND

The parental cell line, 2B4.11 or MA ζΔ66-157, was stimulated with immobilized anti-CD3ε Ab (2C11.145). For antigen stimulation experiments, 2B4.11 or MA ζΔ66-157 cell lines were incubated (5×10^4 cells) with H-2E^k − APC (5×10^5) and the concentrations of PCC p81-104 shown. After 24 h of incubation, the cells were harvested and stained with anti-TCR Vα11 to distinguish them from APC and the percent cell loss was calculated by FACS® analysis.

domains of the CD3 chain to that of the TCR-ζ chain using single chain chimeras. We prepared stably transfected BW cells expressing a Tac chimeric protein containing the cytoplasmic portion of the CD3ε chain that harbors one ITAM motif (TT-ε) and then selected TT-ε and TT-ζ transfectants that had comparable surface expression of the chimeric proteins (Fig. 3 *A*). After stimulation with immobilized anti-Tac mAb for 24 h, significant T cell apoptosis (manifested by PI uptake and a decrease in the PI-negative cells) was observed for the TT-ζ4 clone but very little in the TT-ε5 clone (Fig. 3 *B*). A dose–response comparison showed that between 1 and 3 μg/ml of anti-Tac mAb, greater PCD was observed with the TT-ζ4 clones (maximum 80% cell loss) than with the TT-ε5 clones (maximum 35% cell loss) (Fig. 3 *C*). Table 3 shows several independent TT-ζ clones prepared in our laboratory with different levels of Tac surface expression. In all cases, TT-ζ clones consistently exhibited 50–80% cell loss whereas TT-ε clones only underwent 10–35% cell loss after Tac cross-linking. For both TT-ζ and TT-ε clones, higher Tac surface expression was correlated with greater cell loss. These results show that both the TCR-ζ chain and the CD3ε chain can transmit signals for T cell death. However, when confronted with an equivalent stimulus, chimeras containing the ζ cytoplasmic domain induce apoptosis substantially better than chimeras containing the ε cytoplasmic domain.

Efficient T Cell Apoptosis Requires the ITAM Motifs in the TCR-ζ Chain. To develop further insight into the role of the TCR-ζ chain in apoptosis, we prepared stable transfectants with mutant single chain chimeras in which phenylalanine was substituted for both tyrosines in the ζ chain ITAMs so that the ITAMS were inactivated either individually or in pairs (Fig. 4 *A*). We made the striking observation that in any construct in which the membrane proximal ITAM (Z1 ITAM) was mutated, the ability to induce apoptosis was lost. The fact that apoptosis was severely impaired even when the second and third ζ ITAMs were intact (construct TTmZ1), suggested an especially important role for the Z1 ITAM. We also found the membrane-distal ITAM (Z3) played a clear contributory role since mutations in it always decreased the overall level of apoptosis obtained. The second ITAM (Z2) had more variable effects. The TTmZ2 construct revealed that if the Z1 and Z3 ITAMs were intact, apoptosis was not decreased by the loss of Z2 ITAM. On the other hand, if the Z3 ITAM was mutated, alteration of the Z2 ITAM further decreased, but did not abolish, apoptosis (compare the TTmZ2/3 and the TTmZ3 constructs). These results provided evidence that

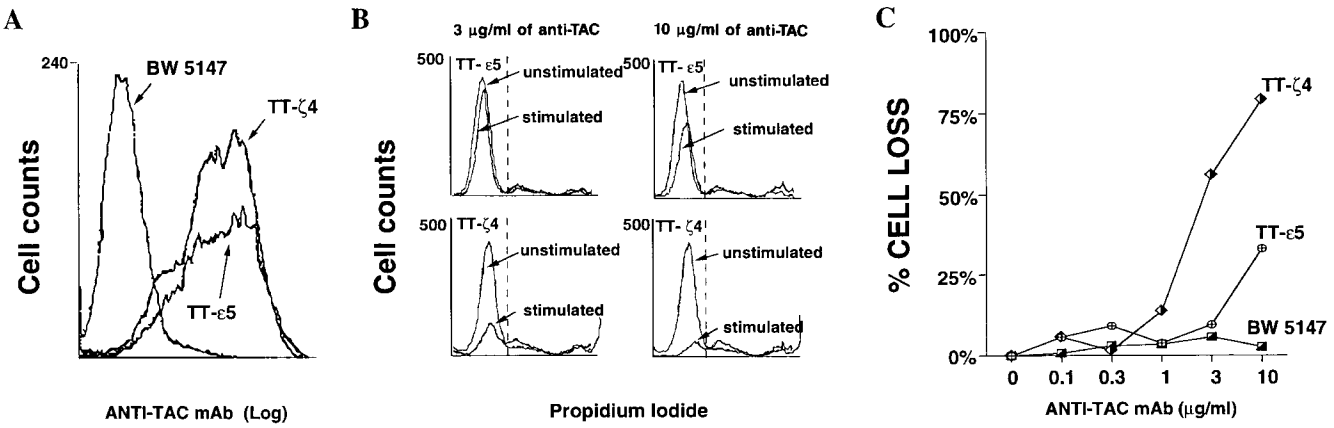

Figure 3. Stimulation by plate-bound anti-Tac mAb induces greater cell death of the TT-ζ4 clone than the TT-ϵ5 clone. (*A*) Flow cytometry histograms of the expression of TT-ζ or TT-ϵ chimera as determined by staining with PE-labeled anti-CD25 mAb. The BW cell line is shown as a control. The mean fluorescence intensity was 270 for TT-ζ clone, 234 for TT-ϵ clone and 7.8 for BW cells. (*B*) Flow cytometry histograms of TT-ζ and TT-ϵ clones that were unstimulated or stimulated for 24 h with either 3 or 10 μg/ml of anti-Tac mAb. The number of viable cells (gate) was determined after analysis by forward scatter profile and PI exclusion. Cells with low PI fluorescence are viable. (*C*) Cell loss calculated after 24 h of stimulation by different concentrations of immobilized anti-Tac mAb. Samples were done in duplicate. The percent cell loss were calculated as described in Materials and Methods.

the ITAM motifs of the ζ chain, particularly the Z1 ITAM, were important for apoptosis. The results also suggested the interesting possibility that individual ITAMs could make different contributions to the overall potency of the ζ chain in stimulating apoptosis, which might imply that each ζ ITAM may have a qualitatively distinct role in signaling.

To more directly demonstrate the different effects of the various ITAM motifs in apoptosis, we compared Tac chimeric proteins containing either the full-length ζ cytoplasmic domain, each ζ ITAM in a single copy, or the ITAMs derived from the CD3 ϵ, γ, or δ chains. The single copy ITAMs were all tested in the context of the CD3ϵ cytoplasmic sequences to eliminate effects caused by the flanking amino acid sequences in each of the TCR chains. We found that greater apoptosis was induced by the full-length ζ chain than by any single ITAM construct (Fig. 4 *B*). Among the single ITAM chimeras, the Z1, i.e., membrane proximal ITAM from the ζ chain, caused substantial apoptosis, whereas the Z3 motif and the CD3ϵ ITAMs triggered a very low but reproducible level of apoptosis. The Z2, CD3γ, and CD3δ ITAMs were incapable of transmitting a death signal. In all cases, stable transfectants that had comparable surface expression of the Tac chimeric molecules were examined, and control experiments showed that all transfectants were susceptible to apoptosis induced by PMA and ionomycin (data not shown). These data support the conclusion that the Z1 ITAM, even when divorced from other sequences in the TCR-ζ protein, maintains its ability to strongly signal for apoptosis. Moreover, the TCR-ζ protein has the quantitatively strongest effect in signaling for apoptosis by virtue of the combination of Z1 with additional ITAMs.

Our observations point to the recruitment of specific ITAM motifs in the transmission of a death signal. Our data

Table 3. *Anti-Tac Stimulation Induces Higher Cell Death in TT-ζ Clones than in TT-ϵ Clones*

Chimera	Clones	Percent Tac$^+$ cells	Fluorescence intensity	Anti-Tac mAb	Percent cell loss	Assay length
		%		*μg/ml*	%	*h*
TT-ζ	clone 4	99	47	10	54.3	24
TT-ζ	clone 7	98	56	10	51.6	24
TT-ζ	clone 4a	98	178	10	79	24
TT-ζ	clone D20p	98	288	5	60	20
TT-ϵ	clone 5	99	50	10	12	24
TT-ϵ	clone 5. b5	99	95	5	20	20
TT-ϵ	clone 5	99	159	10	33	24
TT-ϵ	clone 5. e5	97	203	5	18	20

Different experiments using clones of TT-ζ or TT-ϵ stimulated by various concentrations of anti-Tac mAb as indicated. The percent cell loss were calculated as described in Materials and Methods.

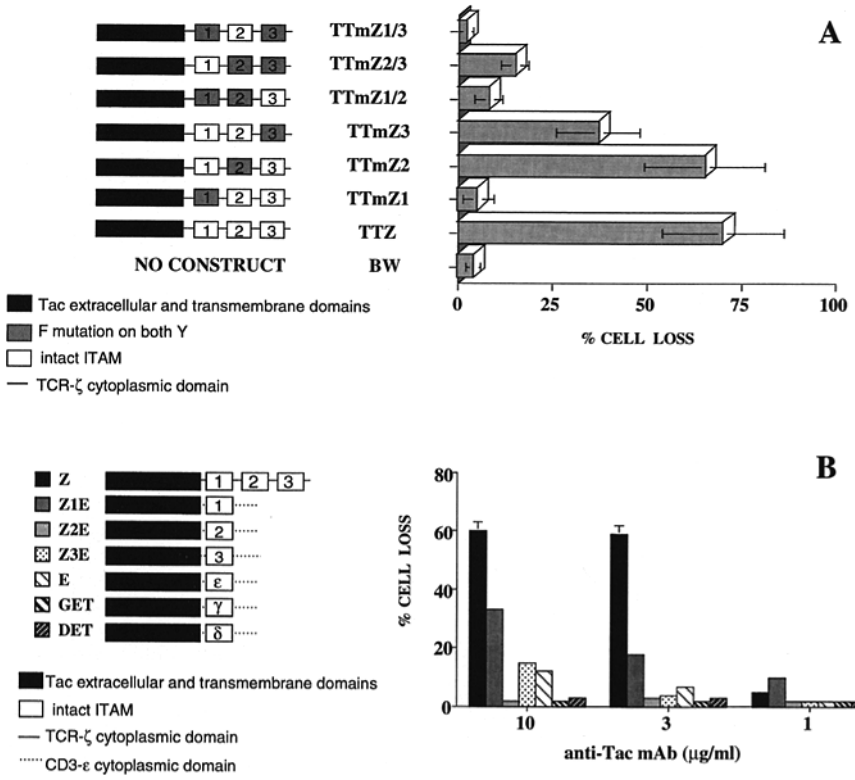

Figure 4. Tyrosine mutations implicate specific ITAM motifs in T cell apoptosis. (*Left*) Structure of chimeric molecules; (*Right*) Histograms of the cell loss after Tac cross-linking as indicated. The Tac extracellular and transmembrane domain are shown as filled rectangles. (*A*) Mutations in the ITAMs of the TCR-ζ chain affect apoptosis. Mutations in each of the TCR-ζ ITAMs: Z1, mutations of Y72, Y83 into F; Z2, mutations of Y111, Y123 into F; and Z3, mutations of Y143, Y153 into F, were constructed either singly or in pairs (*shaded boxes*). Unmutated ITAMs (*open boxes*). Cell loss was calculated by flow cytometry after stimulation by 5 μg/ml of immobilized anti-Tac mAb for 24 h. (*B*) ITAMs from the TCR-ζ or CD3 chains, when substituted for the ITAM of the CD3ϵ chain, have different abilities to signal for apoptosis. The various ITAMs are shown with different shading. Each sample was performed in duplicate and analyzed by FACS®.

suggest a more selective function for different ITAMs than can be inferred from previous analyses of the various TCR chains in triggering activation and IL-2 expression. Recently, Songyang et al. (22, 23) have provided elegant experimental evidence that the three amino acids that lie immediately adjacent to the carbonyl group of phosphorylated tyrosines serve as specificity determinants for the docking of different SH2 domain signalling proteins. In light of their observations, it is important to note that these key residues differ between the Z1 ITAM and the other ITAMs that are present in the oligomeric TCR complex (Table 1). It may be significant that the Zap70 signaling kinase associates more strongly with Z1 ITAM than with the Z2, Z3, or ϵ ITAMs (24). However, our data could also imply that the signaling pathways engaged by the TCR to promote apoptosis differ from those involved in other T cell responses. Further studies will be directed at uncovering the nature of the signals that initiate activation and apoptosis. Elucidation of the Z1 ITAM signaling mechanism will enhance our molecular understanding of the propriocidal apoptosis of mature T cells that preserves lymphocyte homeostasis and peripheral tolerance (5, 40, 45).

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