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# T-cell and basophil activation through the cytoplasmic tail of T-cell-receptor ζ family proteins

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ABSTRACT **The ζ chain of the T-cell antigen receptor is the prototype of a family of proteins that exist as disulfide-linked dimers and are subunits of the T-cell antigen receptor and both IgE and IgG binding Fc receptors. Two related genes encode the ζ and γ proteins. In this study we examine the ability of chimeric proteins consisting of the extracellular domain of the α chain of the interleukin 2 receptor (Tac) and the cytoplasmic domain of either ζ or γ to activate cells when expressed in either T cells or rat basophilic leukemia cells. The ζ and γ chimera were effective at eliciting interleukin 2 production in T cells and serotonin release in rat basophilic leukemia cells when externally cross-linked. Cytoplasmic-tail deletion mutants of ζ and γ were constructed and used to verify the specificity of cell activation by these chimeric proteins. Signaling potencies of complementary mutants having the ζ tail truncated in position 108 or deleted from positions 66 through 114 suggested the presence of several functional domains in ζ.**

The recognition of a specific antigen by T cells is mediated by immunoglobulin-like subunits of the T-cell antigen receptor (TCR) (1, 2). This recognition results in the activation of one or more tyrosine kinases, which in turn are responsible for the phosphorylation of a number of intracellular substrates (3–11). The TCR is a multicomponent surface complex composed of the products of at least six genes (1, 12). Most receptors utilize αβ heterodimers as their antigen-recognition subunits. These are noncovalently linked to CD3 proteins. Three CD3 components have been defined, δ, γ, and ε. Current data point to their presence as dimers of either δε or γε in the mature receptor (13–15). The receptor complex is completed by the presence of a covalently linked dimer called ζ (12, 16).

We have been interested in the role of the ζ chain for a number of reasons. Perhaps most intriguing is the fact that ζ was the first characterized member of a family of proteins. An isoform of ζ, η, represents an alternately spliced form of the ζ gene transcript (17, 18). Identification of the subunit structure of the high-affinity Fcε receptor led to the recognition of a distinct gene, closely related to ζ, which was referred to as the γ chain of this Fc receptor (19). It was then recognized that the ζ chain is not limited to the TCR but can be found as part of the FcγRIII receptor in natural killer cells (20–22). Finally, T cells were identified that expressed both ζ and γ and it was demonstrated that these related proteins can form multiple homo and heterodimers (23). Thus ζ family proteins form dimers that can be components of multiple receptors that can engage in signal transduction in a variety of myelopoietic cells.

The identification of ζ-deficient T cells allowed us to begin to examine the function of this protein (24). In the absence of ζ, the remaining subunits of the receptor assembled in and left the endoplasmic reticulum but failed to reach the surface. Introduction of the cDNA encoding the ζ chain restored surface expression and the reconstituted receptors were able to function (25, 26). Surface expression could be reconstituted with altered cDNAs encoding truncated forms of the protein (27). These mutated receptors were, however, markedly deficient in their ability to respond to antigen. Recently, two groups have directly tested the function of the cytoplasmic tails of ζ proteins by constructing chimeric proteins between either CD8 or CD4 and ζ (28, 29). CD8-ζ chimeras, when expressed in Jurkat T cells were shown to effectively mimic the activation of those cells by way of the full TCR. CD4 chimeras containing the cytoplasmic tail of ζ, η, or γ, when expressed in cytotoxic T cells, could direct the killing of cells that possess targets for the extracellular domain of CD4. In this present study, we report on our use of similar chimeras, using the extracellular domain of the α chain of the human interleukin 2 (IL-2) receptor to investigate several characteristics of the signaling potential of ζ and γ.

## MATERIALS AND METHODS

**Cell Lines and Media.** BW5147 $\alpha^-\beta^-$ cells (here referred to as BW), a variant of BW5147 cells that synthesize no α, β, or CD3 δ or ζ (30) [a gift of W. Born (National Jewish Hospital, Denver)], were cultivated in Dulbecco's modified Eagle's medium (DMEM)/10% (vol/vol) fetal bovine serum/50 μM 2-mercaptoethanol/gentamicin (0.15 mg/ml). Rat basophilic leukemia (RBL) cells (RBL-2H3) [supplied by O. Letourneur (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD)] were cultivated in DMEM/20% fetal bovine serum/50 mM Hepes, pH 7.3/gentamicin (0.15 mg/ml).

**Antibodies.** The following antibodies were used: 145-2C11, a hamster monoclonal antibody (mAb) recognizing the CD3 ε chain (31); 33B3.1, a rat mAb directed against Tac (AMAC, Westbrook, ME); 7G7, a mouse mAb directed against Tac (32); G7, a rat mAb binding the Thy-1 molecule (33); F23.1, a mouse mAb recognizing TCR β chains encoded by the β-chain variable-region family Vβ8 (34). Anti-dinitrophenyl (DNP) IgE was from the hybridoma Hi DNP ε 26.82, kindly provided by H. Metzger (National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD) (35). Polyclonal goat anti-rat immunoglobulin was obtained from Boehringer Mannheim. Fluorescein-conjugated rabbit anti-rat and goat anti-mouse immunoglobulins were purchased, respectively, from Cappel Laboratories and Kirkegaard and Perry Laboratories.

**Chimera Construction and Transfection.** All chimeras were constructed by the polymerase chain reaction (PCR) (36). PCR products were directly cloned into the expression vector pCDLSRα (37) and confirmed by complete DNA sequencing. For transient expression, the DEAE-dextran method (38) was used (10 μg of DNA for $1 \times 10^7$ cells). Stable transfectants were obtained by electroporation of BW cells with 18 μg

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Abbreviations: IL-2, interleukin 2; mAb, monoclonal antibody; RBL, rat basophilic leukemia; TCR, T-cell antigen receptor; endo H, endoglycosidase H.

of plasmid linearized by *Hin*dIII and 2 μg of pFneo cut by *Eco*RI per $1 \times 10^7$ cells. A voltage of 290 V and a capacitance of 250 μF were applied by a Bio-Rad Gene Pulser. Twenty-four hours after transfection, cells were plated out in 24-well plates ($5 \times 10^4$ cells per ml) in Geneticin-containing medium (3 mg/ml; GIBCO/BRL). Electroporation of RBL cells was performed using a voltage of 250 V and a capacitance of 500 μF. After 1 week of culture in medium containing Geneticin (1 mg/ml), clones were obtained by limiting dilution.

**Metabolic Labeling.** Transiently transfected cells were pulse-labeled for 20 min at 37°C with [$^{35}$S]methionine at 0.5 mCi/ml (Tran$^{35}$S-label, ICN; 1 Ci = 37 GBq). Cells were solubilized at 4°C in Triton X-100 lysis buffer as described (5). Tac chimera proteins were isolated with mAb 7G7 (31) and analyzed by SDS/PAGE on 10% polyacrylamide gels. Endoglycosidase (endo H) treatments were performed as described (39).

**Stimulation of BW Transfectants with Antibodies.** In all experiments, $1 \times 10^5$ cells were cultured in duplicate in 96-well flat-bottomed plates in a final volume of 200 μl. Anti-Tac stimulation was performed using plates precoated with 50 μl of phosphate-buffered saline (PBS) containing various concentrations of mAb 33B3.1. For Thy-1 activation, cells were directly incubated with soluble mAb G7 (1:50 dilution of ascites fluid). Supernatants were harvested after 24 hr of culture and the IL-2 content was determined (40).

**Serotonin Release.** Transfected RBL cells were cultured 24 hr at $2 \times 10^5$ cells per ml with 5-[1,2-$^3$H(N)]hydroxytryptamine binoxalate (serotonin) (New England Nuclear) at 2 μCi/ml in 24-well plates (1 ml per well). Cells were washed once with medium and incubated for 1 hr at 37°C with anti-dinitrophenyl IgE at 10 μg/ml or various dilutions of mAb 33B3.1. After washing twice, cells were incubated at 37°C for 45 min with antigen (dinitrophenyl-conjugated bovine serum albumin) at 1 μg/ml or goat anti-mouse immunoglobulin at 10 μg/ml. [$^3$H]Serotonin release was stopped by adding ice-cold PBS and, after centrifugation, [$^3$H]serotonin in supernatants was measured using a Packard liquid scintillation counter. Determinations were done in triplicate and results were expressed as the percentage of specific release (spontaneous serotonin release minus induced serotonin release divided by total serotonin stored in the cells).

## RESULTS

**Expression of Chimeric Proteins.** Chimeric proteins were constructed to include the extracellular domain of the α chain of the human IL-2 receptor (the Tac antigen) and the transmembrane domains from either Tac or ζ. The cytoplasmic domains of the chimeras derived from the murine ζ or γ chains. The transmembrane region of ζ is sufficient to produce disulfide-linked dimers and, therefore, we have utilized this region to compare monomeric with dimeric chimeras. A chimera between Tac and ζ utilizing the Tac transmembrane domain is abbreviated TTζ, and Tζζ refers to the chimera containing the ζ transmembrane domain. Alterations in the cytoplasmic domains were produced by truncating the reading frames by the introduction of stop codons. These truncated constructs are denoted by the number of the amino acid that has been changed to form such a stop codon. The sequences of the cytoplasmic tails and the location of the deletions are shown in Fig. 1*A*. For functional experiments, the cDNA constructs were stably expressed in a variant of BW5147 cells (hereafter referred to as BW cells) that synthesizes no α, β, or CD3 δ or ζ chains and expresses no TCR on its surface (30). To assess the protein products made, transiently transfected cells were pulse-labeled with [$^{35}$S]methionine and cell lysates were immunoprecipitated with an anti-Tac mAb (7G7). Shown in Fig. 1*B* is a pulse–chase experiment performed on TTζ demonstrating the efficient

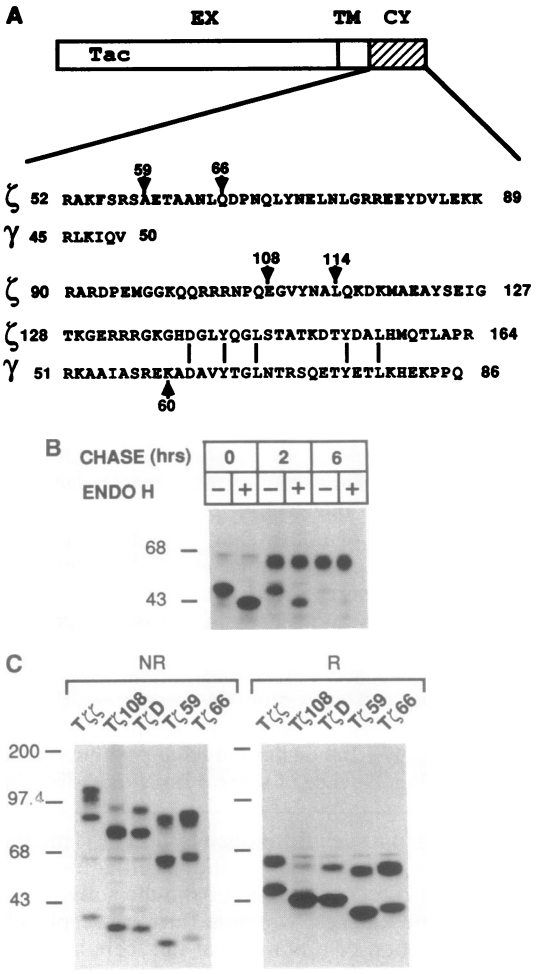


FIG. 1. (*A*) Schematic representation of the chimera containing Tac external (EX) and transmembrane (TM) domains and the cytoplasmic domains (CY) of ζ and γ chains. Sites of truncation or deletion are indicated by arrows and numbers of the amino acids that have been changed in stop codons or substituted. A chimera between Tac and ζ utilizing the Tac transmembrane domain is abbreviated TTζ and Tζζ refers to the chimera containing the ζ transmembrane domain. Truncated constructs are denoted by the number of the amino acid that has been changed to a stop codon (e.g., Tζ66). The internal deletion of the ζ tail, which lacks amino acids 66–114, is denoted TζD. (*B* and *C*) Biosynthesis of some chimeric proteins expressed transiently in BW cells. (*B*) Pulse–chase experiment in cells transfected with TTζ. Immunoprecipitates were either not treated (−) or treated (+) with endo H. (*C*) Biochemical characterization of some chimeric constructs. Transfected cells were pulse-labeled for 1 hr with [$^{35}$S]methionine. Immunoprecipitates with anti-Tac antibody (7G7) were analyzed in nonreduced (NR) or reduced (R) SDS/polyacrylamide gels. The positions of the molecular mass markers are shown to the left in kDa.

processing of the Tac carbohydrate side chains. No significant loss of protein is observed over 6 hr. In Fig. 1*C* are examples of dimeric constructs analyzed under either nonreducing or reducing conditions. After a 1-hr pulse, the two forms seen under reducing conditions represent unprocessed (endo H sensitive; lower bands) and Golgi-processed (endo H resistant; upper bands) carbohydrates on the Tac extracellular domain. Under nonreducing conditions, the lower bands correspond to monomeric forms of the chimeras (the nonreduction of intradisulfide bonds in the Tac external domain results in a migration different from what is observed under reducing conditions), whereas the two upper bands represent unprocessed and processed dimers. Note that relatively little of the monomeric protein (Tζζ) demonstrates Golgi processing. Cell surface expression of representative stable clones of

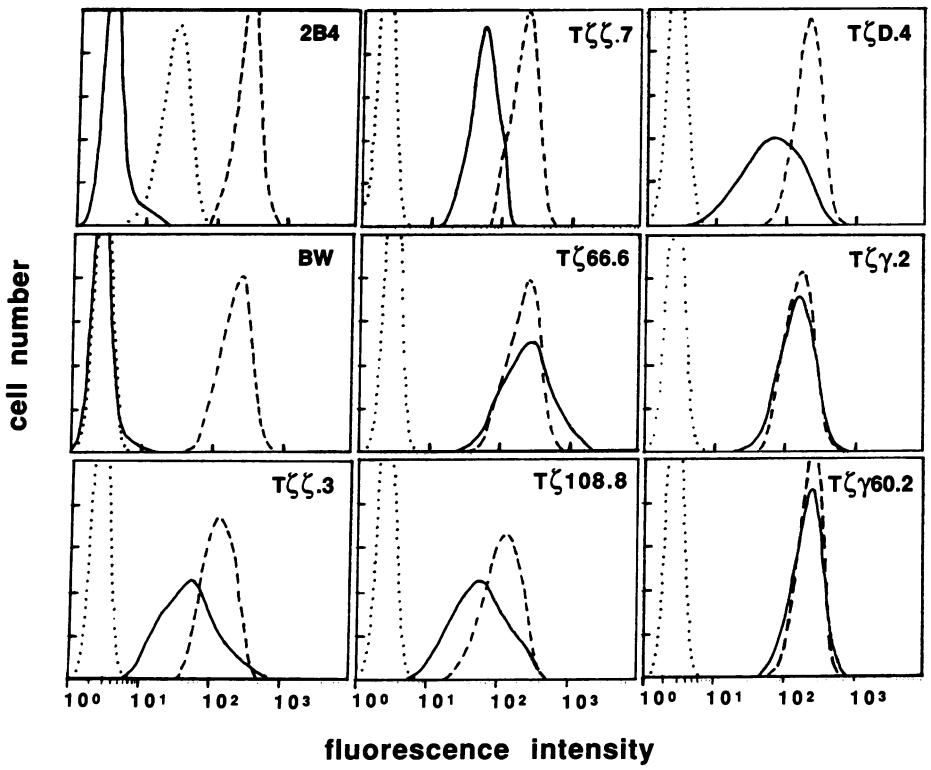


FIG. 2. Flow cytometry analysis of BW cells stably transfected with the various chimeras. The indicated cells were stained with an anti-CD3ε mAb (2C11) (dotted line), an anti-Thy-1 mAb (G7) (dashed line), or an anti-Tac mAb (33B3.1) (solid line) followed by labeling with fluorescein-conjugated rabbit anti-rat immunoglobulin.

the chimeras were assessed by flow cytometry using the 33B3.1 (anti-Tac) mAb (Fig. 2). No surface expression of the endogenous CD3 ε and γ chains was detected in any of these cells by flow cytometry using an anti-ε mAb (2C11). These chimeric proteins are not capable of interacting with other components of the TCR and fail to rescue surface expression of ζ-negative T cells (data not shown). Thy-1 expression showed little variation from clone to clone.

**Stimulation of IL-2 Production.** The transfected cells were stimulated with an anti-Tac antibody precoated on 96-well plates and the supernatants were examined for IL-2 activity (Fig. 3*A*). High levels of IL-2 (700–1400 units/ml) were produced by cross-linking the chimera containing the native ζ tail. No difference was noted when we compare constructs containing either the Tac or ζ transmembrane domain. No IL-2 is detected in the absence of cross-linking of the anti-Tac mAb. All of these transfectants were able to be stimulated to produce IL-2 by the addition of phorbol esters and calcium ionophore that elicited approximately two times the maximal IL-2 produced by the anti-Tac cross-linking. Stimulation with mAb 2C11 (anti-CD3ε) did not result in IL-2 production.

We next examined structural alterations in the ζ tail by comparing the full-length constructs with two truncations (108 and 66) and one internal deletion that lacked amino acids 66–114 (TζD). The most proximal truncation yielded no IL-2 production whereas the more distal truncation and the internal deletion showed an ≈1.5-order-of-magnitude shift in the anti-Tac dose–response curve (Fig. 3*A*). The more distal deletion is comparable to mutations that abrogated antigen responsiveness when ζ-negative T-cell hybridomas were reconstituted with altered ζ subunits (27). In those previous studies, we noted that the loss of antigen response with mutations in ζ was paralleled by a loss in the response to the stimulation of the cells with an antibody (G7) directed against the Thy-1 molecule (33). All of the transfectants expressed equivalent levels of Thy-1 and we were thus interested in determining whether the expression of these ζ chimeras resulted in responsiveness to Thy-1 stimulation. Even in receptor-positive T cells this Thy-1 antibody yields maximal levels of IL-2 well below that achieved with direct receptor stimulation. G7 stimulation failed to induce detectable IL-2 in cells transfected with either Tac or the monomeric TTζ chimera. However, stimulation of cells expressing the Tζζ construct, resulted in detectable levels of IL-2 (Fig. 3*B*). None of the truncations of ζ or the γ tail supported any detectable IL-2 production. In contrast, the internal deletion mutant did give detectable IL-2 levels after the addition of the Thy-1 antibody.

**Comparison of ζ with γ.** Finally, we compared the ability of the cytoplasmic tails of ζ and γ in these activation assays. Despite the fact that two times more Tζγ than Tζζ chimera was expressed in the BW cells (Fig. 2), the ζ tail was three times more effective (comparing maximal IL-2 release) than the γ tail (Fig. 3*A*). Furthermore, the dose–response curve showed a shift of about 1 order of magnitude between Tζγ and Tζζ transfectants. IL-2 production by the Tζγ could be abrogated by a cytoplasmic truncation of γ at amino acid 60. As γ was initially described as part of the high-affinity Fcε receptor, we wondered whether the cytoplasmic tail of γ would be relatively more effective at activating responses normally coupled to the IgE receptor (41). To assess this, we transfected RBL cells with either TTζ or TTγ and examined the ability of Tac cross-linking to release serotonin. When comparable levels of these chimeras were expressed (Fig. 4), anti-Tac cross-linking resulted in serotonin release for both constructs (Fig. 5). Nevertheless, the γ chimera was less potent at eliciting serotonin release, as a 2-order-of-magnitude shift in the anti-Tac dose–response curve was noted. When those clones were stimulated with IgE and antigen, ≈50% of the total serotonin associated with the cells was released. Like IL-2 production in BW cells, the serotonin release due to the tails of either ζ or γ could be abrogated by truncating either of these cytoplasmic domains (Fig. 5). It has been shown (42) that the transmembrane domain of γ is

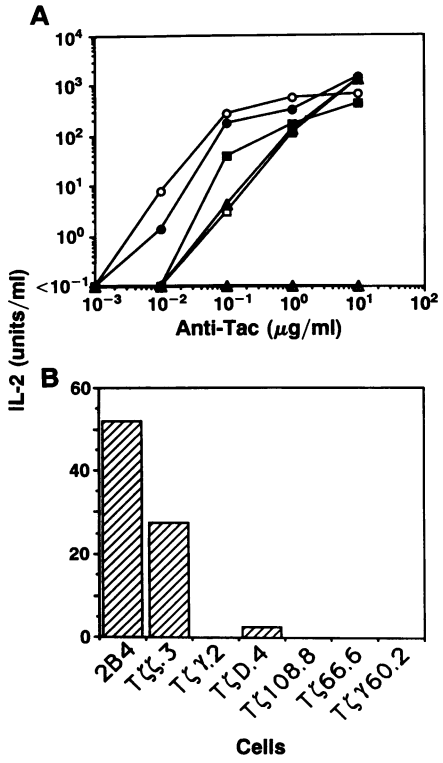


FIG. 3. (*A*) IL-2 production of stable BW transfectants in response to anti-Tac (33B3.1) mAb stimulation. ●, Tζζ clone 3; ○, Tζζ clone 7; ■, Tζγ clone 2; □, TζD clone 4; ▲, Tζ108 clone 8; △, Tζ66 clone 6; ◆, Tζγ60 clone 2. (*B*) Restoration of responsiveness to Thy-1 stimulation by expression of ζ chimeras. BW transfectants were stimulated for 24 hr with soluble anti-Thy-1 mAb (1:50 dilution of G7 ascites fluid). Supernatants were then assayed for IL-2 content.

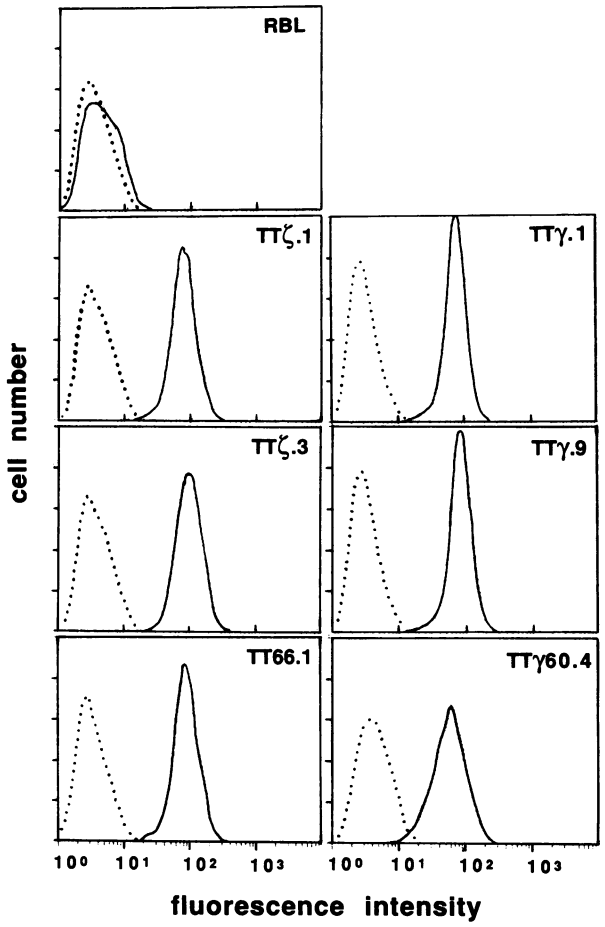


FIG. 4. Cell surface expression of the various chimeras transfected in RBL cells. The indicated cells were stained with either an anti-Vβ8 mAb (F23.1) (dotted lines) as a negative control or an anti-Tac mAb (7G7) (solid lines) followed by staining with fluorescein-conjugated goat anti-mouse polyclonal immunoglobulin.

responsible for its assembly with the other subunits of the FcεR receptor. Thus the use of these monomeric chimeras would not be expected to result in interactions with these other surface components.

## DISCUSSION

These studies demonstrate a number of properties of the cytoplasmic tails of ζ and FcεRIγ. These proteins normally exist as subunits of complex multicomponent surface receptors. Despite the complexity of their native receptors, it is clear that they alone appear to be capable of initiating signals that can lead to cellular activation events in either T cells (IL-2 production) or RBL cells (serotonin release). These findings support and extend the observations of Irving and Weiss (28) and Romeo and Seed (29). In the former study, external cross-linking of a CD8–ζ chimera resulted in the stimulation of tyrosine phosphorylation and several of the characterized ensuing biochemical events of TCR-mediated cell activation. Using the constructs reported in this study, we first assessed the effect of alterations in the cytoplasmic domains of ζ and γ FcεRI tails. The ability to abolish activation by truncations of these cytoplasmic tails (position 66 in ζ and position 60 in γ) establishes this expression–activation system as useful for defining more precisely the structural requirement for ζ and γ function. Two complementary chimeras, TζD (ζ tail internal deletion from positions 66 to 114) and Tζ108 (ζ truncation at position 108) resulted in reduced IL-2 production in comparison with the full-length ζ tail, suggesting at least two functional domains in the ζ tail.

Interestingly, chimeras containing the ζ transmembrane domain were able to support IL-2 production in response to the anti-Thy-1 antibody G7. We ruled out that this domain alone supported Thy-1-mediated activation by expression of a TζT chimera, which was inactive. Furthermore, a Tζγ chimera, expressing the ζ transmembrane domain, was unable to reconstitute Thy-1 responsiveness. How Thy-1 stimulation utilizes the TCR or the ζ chimeras to activate the cell is unknown as the protein is attached to the membrane through a phosphatidylinositol-glycan linkage (43).

The ζ tail function in these chimeras can be contrasted with our previous findings on the reconstitution of TCR complexes with full-length or truncated versions of ζ (30). In those studies, the truncation of ζ at amino acid 107 resulted in a reconstituted surface receptor that was severely impaired in

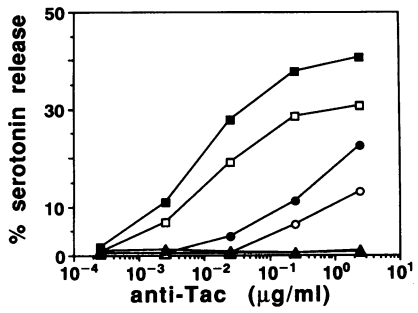


FIG. 5. Comparison of the ability of the cytoplasmic tails of ζ and γ to support the release of serotonin in RBL cells upon anti-Tac mAb stimulation. ■, TTζ clone 3; □, TTζ clone 1; ●, TTγ clone 9; ○, TTγ clone 1; ▲, TTγ60 clone 4; △, TT66 clone 1.

its ability to respond to either antigen or Thy-1 stimulation. Direct cross-linking of the analogous Tac–$\zeta$ chimera resulted in diminished but definite IL-2 production. In the context of the full receptor, we detected no IL-2 production to antigen. In contrast when this reconstituted TCR was externally cross-linked, IL-2 production was indistinguishable from a TCR reconstituted with a full-length $\zeta$. Perhaps the anti-Tac cross-linking of the chimera mimics the direct cross-linking of the TCR by antibodies but not antigen triggering. The functional capacity of the altered $\zeta$ tail might reflect simply the nature or extent of the cross-linking. Alternatively, this truncated $\zeta$ tail when part of a full TCR might be inactive. In this case, other chains of the TCR would be predicted to function in response to external cross-linking. This is supported by the observation that in T-cell hybridomas that express no detectable $\zeta$ chain, external cross-linking of the limited number of receptors that are expressed on the cell surface results in IL-2 production (25, 26). The direct signaling potential of other TCR chains awaits testing, perhaps using chimeras such as those studied for $\zeta$. Preliminary results using a TT$\varepsilon$ chimera suggest that CD3–$\varepsilon$ alone can activate T cells (unpublished data).

It is not surprising that both $\zeta$ and $\gamma$ can mediate intracellular signaling events. Perhaps more interesting is the fact that the $\zeta$ tail is more efficient than the $\gamma$ tail even in RBL cells. Besides the $\gamma$ chain, some other Fc receptor components may have complementary signaling properties. Both the effect of truncations and the sequence conservation between $\zeta$ and $\gamma$ point to the carboxyl-terminal region of the two proteins as most critical to function in the assays reported here. Establishing the precise motif(s) in these proteins required for signaling may point to functional sequences in other less closely related proteins, such as subunits of the B-cell antigen receptor. The ability of the cytoplasmic tails of $\zeta$ and Fc$\varepsilon$RI$\gamma$ to mediate cellular activation presumably underlies their incorporation as subunits of at least three receptors defined to date, the TCR, Fc$\varepsilon$RI, and Fc$\gamma$RIII. Our current views of TCR activation point to the coupling to src family tyrosine kinases as the essential events in the signaling through this receptor (44). A similar paradigm may underlie activation by signaling Fc receptors and the B-cell antigen receptor. Thus the $\zeta$ family (and other $\zeta$-related proteins) may represent a type of membrane signaling molecule most likely involved in the interaction with src family tyrosine kinases that have been implicated for both the TCR (7, 45) and the Fc$\varepsilon$RI (46).

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