Brief Definitive Report

The Pre–T Cell Receptor (TCR) Complex is Functionally Coupled to the TCR-ζ Subunit

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Summary

The pre–T cell receptor (TCR) complex regulates early T cell development and consists of a heterodimer of the TCR-β subunit in association with the pre–TCR-α chain. Notably, in contrast to α/β-expressing T cells, several studies suggested that the TCR-ζ chain is not stably associated with this pre-TCR complex. To examine the proximal signaling processes mediated by the pre-TCR complex and the role of the TCR-ζ chain in these processes, we stimulated pre-TCR–expressing cells and analyzed the interactions of the TCR/CD3 invariant chains with the Syk/ZAP-70 family of protein tyrosine kinases. Stimulation of the pre-TCR complex led to the tyrosine phosphorylation of the CD3ε and TCR-ζ chains, as well as the phosphorylation and association of ZAP-70 and Syk with phosphorylated CD3ε and TCR-ζ. These results demonstrate that the pre-TCR complex is functionally coupled to the TCR-ζ subunit and to the ZAP-70 and Syk protein tyrosine kinases.

The development of a TCR-α/β–expressing cell in the thymus follows a precisely ordered sequence of events initiated by the successful rearrangement and expression of the TCR-β gene (1). The surface expression of the TCR-β chain, in the absence of the TCR-α subunit, regulates early T cell development by promoting the transition from $CD4^-CD8^-$ to $CD4^+CD8^+$ cells and the expansion of both subsets (2–6). These developmental events are regulated by the pre-TCR (von Boehmer, H., unpublished results), a multisubunit receptor complex that includes the TCR-β chain covalently associated with the pre–T α chain (pTα) (7, 8). The murine pTα chain is a type I transmembrane protein with a single extracellular Ig-like constant region domain and a 31-residue cytoplasmic tail (7).

The surface expression of the TCR-β chain in the absence of the TCR-α subunits has been reported in two immature T cell lines (3, 8, 9), in TCR-β–transgenic rearrangement-deficient mice (2–6), and in TCR-α–deficient mice (4, 8). In TCR-β–transgenic mice, the β chain was found as a glycophosphatidylinositol-linked monomer (3, 6), as well as a dimer that was also found in normal mice (7, 10). Initial biochemical studies suggested that the β chain was expressed as a homodimer (3), but it is now apparent that the β chain forms a heterodimer with the pTα subunit (7, 8). Interestingly, the TCR-β–pTα complex maintains a significantly weaker, if any, biochemical association with the TCR-ζ chain when compared with the TCR-α/β complex (3).

The signal transduction functions of the TCR-α/β complex are localized in a common signaling motif present in the CD3 and TCR-ζ chains (11). This sequence, termed immune receptor tyrosine-based activation motif (ITAM), is present as a single copy in CD3γ, -δ, and -ε, and as three copies in TCR-ζ. The pre-TCR complex is also capable of transducing intracellular signals after receptor ligation, as assessed by calcium mobilization (10). In view of the altered composition or subunit associations of the pre-TCR complex with the TCR-ζ chains, it is possible that the pre-TCR complex couples to downstream signal transduction pathways differently than the TCR-α/β complex. For example, the presence of fewer ITAMs in the multimeric pre-TCR complex may result in decreased signal amplification after TCR engagement (12). In addition, individual ITAMs may couple to distinct intracellular signaling molecules, which is consistent with several reports that the TCR-α/β complex comprises at least two distinct signal-transducing modules (13, 14). Mice that are rendered deficient in TCR-ζ expression are able to generate very small numbers of single-positive thymocytes (15–18). However, the TCR complexes expressed on the $CD4^+CD8^+$ thymocytes in these ζ-deficient mice can transduce intracellular signals, suggesting that the CD3 chains alone are functioning as the transduction modules (18). Thus, it is not clear whether the TCR-ζ chain is functionally coupled to the pre-TCR. We have used a pre-TCR–expressing cell line SCB.29 to examine the proximal intracellular signaling

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functions of the pre-TCR complex and to determine whether the TCR-ζ chain is involved in these signaling processes.

Materials and Methods

Cell Lines and Animals. The Scid mouse–derived immature T cell line (SciET27F) and the TCR-β–transfected derivative of this cell line (SCB.29) have been described in detail elsewhere (3). The SCB.29 cell line expresses the pre-TCR complex (3, 8). The SCαpβH.8 (SCαβ) cell line is a TCR-α/β–expressing derivative of the SciET27F line. Murine thymocytes and LN cells were isolated as previously described (19).

Antibodies and Antisera. The antibodies used for immunoprecipitation, immunoblotting, and FACScan® (Becton Dickinson & Co., Cockeysville, MD) analyses are as follows: 145-2C11, CD3ε (American Type Culture Collection [ATCC], Rockville, MD); GK1.5, CD4 (ATCC); 53-6.7, CD8 (ATCC); 4G10, phosphotyrosine (Upstate Biotechnology, Inc., Lake Placid, NY); G3, TCR-ζ chain (20). Fluorescein-conjugated anti-CD8α and PE-conjugated anti-CD4 were purchased from Collaborative Biomedical Products (Bedford, MA). PE-conjugated anti-CD3ε (145-2C11) was obtained from PharMingen (San Diego, CA). Horseradish peroxidase–conjugated goat anti–mouse IgG and goat anti–rabbit IgG were purchased from Southern Biotechnology, Inc. (Birmingham, AL); ^{125}I-protein A was purchased from Amersham Corp. (Arlington Heights, IL); and alkaline phosphatase–conjugated goat anti–rabbit Ig was obtained from Bio-Rad Laboratories (Richmond, CA). An mAb (6B10.2) directed against the cytoplasmic domain of human TCR-ζ (CDKRGRDPEMGGKPRRKN) was generated using standard hybridoma technology (20). The cysteine at the NH_2 terminus of this peptide was included to facilitate coupling to keyhole limpet hemocyanin. The mAb is capable of precipitating both nonphosphorylated and tyrosine-phosphorylated forms of the TCR-ζ chain. A polyclonal rabbit anti–ZAP-70 peptide antisera (1222) was used for immunoprecipitation and immunoblotting experiments as previously described (19). A polyclonal rabbit anti-Syk antisera was generated against a peptide sequence (amino acids 314–339) of the human Syk protein tyrosine kinase (PTK). Rabbit antisera against a keyhole limpet hemocyanin–Syk peptide conjugate were generated as described (21).

Stimulation, Precipitation, and Immunoblotting of the Pre–T Cell Lines. The SciET27F, SCB.29, and SCαβ were washed several times in PBS and subsequently resuspended in PBS at a concentration of 1×10^8 cells/ml. Cells were stimulated at 37°C for the indicated times with 10 μg/ml of anti-CD3ε (145-2C11), rapidly pelleted, and lysed at a concentration of 1×10^8 cells/ml in a Triton X-100–containing lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2.0 mM EDTA, and protease and phosphatase inhibitors). For CD3ε precipitates, $4–6 \times 10^7$ cells were lysed in 0.5% Triton X-100 to maintain TCR-ζ associations as described elsewhere (22). 1% Triton X-100–containing lysis buffers were used for ZAP-70, TCR-ζ, Syk, and normal rabbit sera (NRS) immunoprecipitates. Protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and goat anti–rabbit Ig agarose beads (Sigma Chemical Co., St. Louis, MO) were used to precipitate the mAbs and polyclonal rabbit antisera, respectively. Immunoprecipitates were analyzed as described elsewhere (19). For comparative immunoblotting, lysates from the different cell populations were prepared, and the protein content of each preparation was subsequently determined with a protein assay kit (Bio-Rad Laboratories). Equivalent amounts of protein were directly resolved by SDS-PAGE and analyzed as described in the figure legends. In vitro kinase assays were performed essentially as described (23). After transfer to polyvinylidene difluoride (PVDF) membranes, the membranes were treated in 1 M KOH for 1 h at 58°C to remove any phosphate present on serine or threonine residues. The membranes were subsequently washed and assayed by autoradiography.

Results and Discussion

The pre-TCR–expressing cell line SCB.29 is capable of transducing intracellular signals after receptor ligation (3). To further characterize the signal transduction processes mediated by the pre-TCR, we analyzed the pre–T cell lines for the expression of several proteins implicated in proximal TCR signaling events (24). Lysates were prepared from the parental cell line (SciET27F), the pre-TCR–expressing cells (SCB.29), a TCR-α/β–expressing immature cell line (SCαβ), and murine thymocytes and LN T cells. Equivalent amounts of protein were resolved on SDS-PAGE and immunoblotted with antibodies against ZAP-70, Syk, Lck, Fyn, and the TCR-ζ subunit. In all three immature T cell lines analyzed, ZAP-70 and Lck were expressed at levels similar to those found in thymocytes (Fig. 1). In contrast, the Syk PTK was only weakly expressed in the immature cell lines, approximating the levels detected in murine LN T cells. The levels of the Fyn PTK appeared more variable between the different cell lines. Consistent with earlier reports, the TCR-ζ chain was ex-

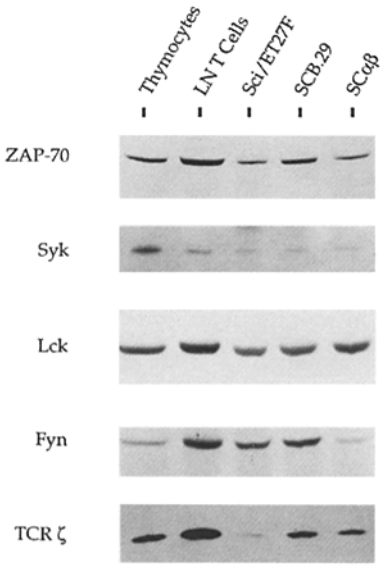

Figure 1. The pre–T cell lines express all the components required for proximal TCR signaling process. Lysates were prepared from thymocytes, LN T cells, the parental cell line (SciET27F), the pre-TCR–expressing derivative (SCB.29), and the TCR-α/β–expressing pre–T cell lines. Equivalent amounts of protein were immunoblotted for ZAP-70, Lck, and Fyn. For Syk and TCR-ζ immunoblots, the cell lysates were initially immunoprecipitated with polyclonal anti-Syk antisera or an mAb against TCR-ζ, respectively.

pressed in all the pre–T cell lines examined, although it appeared to be low in the parental SciET27F cells (3). In summary, the different pre–T cell lines expressed all the proteins implicated in proximal TCR signaling processes (24).

Cell lines expressing the pre-TCR complex are capable of mobilizing intracellular calcium after pre-TCR ligation (3, 10). Since it has been difficult to document an association between the pre-TCR complex and TCR-ζ, it was conceivable that the calcium responses elicited in the pre-TCR–expressing cells were independent of TCR-ζ–mediated signals. To examine the signal transduction events in the pre–T cells and to determine whether TCR-ζ was involved in these processes, the pre–T cell line SCB.29 and the TCR-α/β–expressing derivative (SCαβ) were stimulated with an mAb directed against the CD3ε chain. Unstimulated and anti-CD3ε–stimulated cells were then lysed in Triton X-100–containing lysis buffers under conditions that preserve the association of the TCR subunits (22). Specific proteins from the lysates were then immunoprecipitated with antibodies against either CD3ε, ZAP-70, TCR-ζ, or NRS as a nonspecific control. The precipitates were resolved on SDS-PAGE and immunoblotted with either antiphosphotyrosine (4G10) or anti–TCR-ζ mAbs. In unstimulated TCR-α/β–expressing cells, the nonphosphorylated TCR-ζ subunit (16 kD) as well as a weak tyrosine-phosphorylated form of TCR-ζ (apparent molecular weight of 21 kD) coprecipitated with the CD3ε chain (Fig. 2, *A* and *B*, lane *1*). TCR ligation led to an increase in TCR-ζ phosphorylation (phospho-ζ), as demonstrated by the induction of the 21- and 23-kD forms of phospho-ζ, as well as the phosphorylation of a 70-kD coprecipitating protein (lane *2*). This 70-kD phosphoprotein corresponds to the ZAP-70 PTK, as demonstrated by the use of anti–ZAP-70–specific polyclonal antisera (lanes *3* and *4*). Finally, an anti–TCR-ζ–specific mAb is also capable of precipitating the 21- and 23-kD forms of phospho-ζ after TCR engagement (lanes *5* and *6*). The ability of the anti-CD3ε mAb to coprecipitate phospho-ζ and tyrosine-phosphorylated ZAP-70 is consistent with our earlier studies using thymocytes and peripheral LN T cells (19). It should be noted that the intensity of the p23 form of phospho-ζ relative to the p21 form is much higher in the pre–T cells when compared with the levels detected in murine thymocytes and LN T cells (19). In fact, these levels are similar to those found in T cell clones stimulated with agonist peptides (25, 26). The reason for these differences is unknown and requires further analysis.

In marked contrast to the strong association between TCR-ζ and CD3ε in the TCR-α/β–expressing immature T cells, we were unable to detect an association between TCR-ζ and CD3ε in the pre-TCR–expressing cell line SCB.29 under the immunoprecipitation conditions used. No nonphosphorylated TCR-ζ (16 kD) was detected in the CD3ε precipitates, whereas antiphosphotyrosine immunoblots of CD3ε immunoprecipitates obtained from either unstimulated or TCR-stimulated lysates failed to reveal the presence of phospho-ζ (Fig. 2, *C* and *D*, lanes *1* and *2*). Receptor engagement in the pre-TCR–expressing cells did lead to the tyrosine phosphorylation of the CD3ε chain, and to association and tyrosine phosphorylation of ZAP-70 (lane *2*). A weak 21-kD form of phospho-ζ coprecipitated with ZAP-70 immunoprecipitates from lysates of unstimulated SCB.29 cells. More impressively, ligation of the pre-

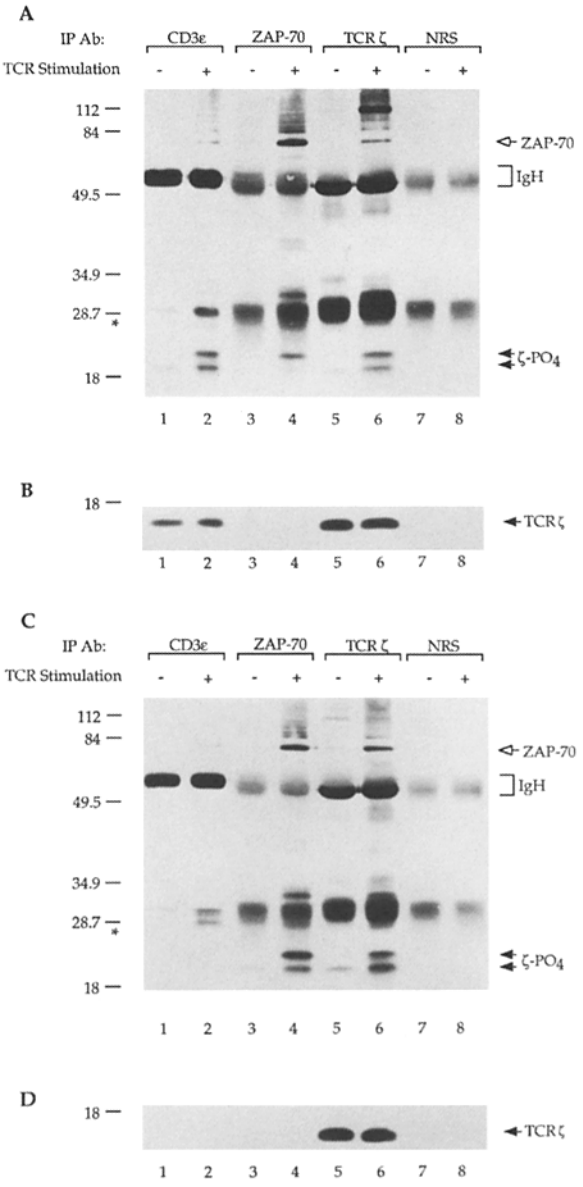

Figure 2. The TCR-ζ chain becomes tyrosine phosphorylated and associates with ZAP-70 after receptor ligation in pre-TCR–expressing cells. The SCαβ (*A* and *B*) and SCB.29 (*C* and *D*) cells (5×10^7 cells/lane) were left untreated (lanes *1*, *3*, *5*, and *7*) or were stimulated with anti-CD3ε mAbs for 3 min (lanes *2*, *4*, *6*, and *8*), were rapidly pelleted, and were subsequently lysed in 0.5% Triton X-100 (lanes *1* and *2*) or 1% Triton X-100–containing lysis buffers (lanes *3–8*). Lysates from the unstimulated and TCR-stimulated lysates were immunoprecipitated with anti-CD3ε mAb (lanes *1* and *2*), anti–ZAP-70 antisera (lanes *3* and *4*), anti–TCR-ζ mAb (lanes *5* and *6*), or NRS as a negative control (lanes *5* and *6*). The precipitates were resolved on 12.5% SDS-PAGE, transferred to PVDF, and blotted with antiphosphotyrosine mAbs (*A* and *C*). The region below 18 kD was blotted with anti-ζ–specific mAbs (*B* and *D*). These results are representative of five independent experiments.

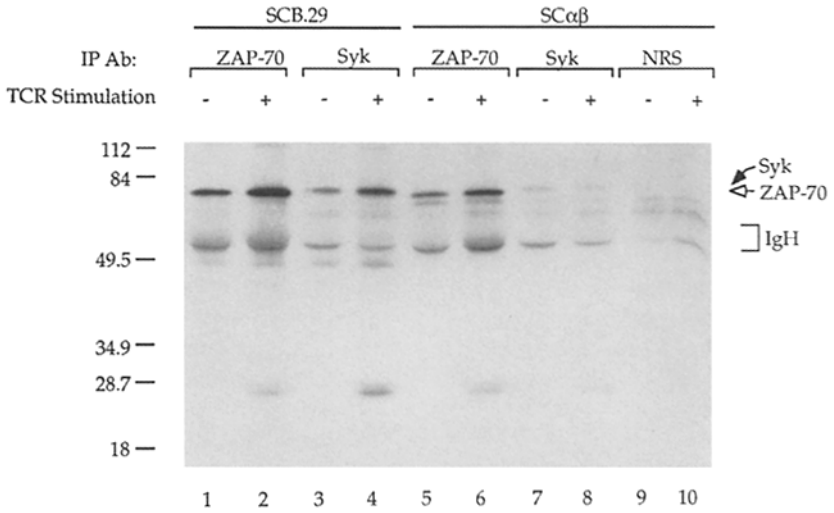

Figure 3. The ZAP-70/Syk family of proteins tyrosine kinases are activated after receptor engagement. ZAP-70 (lanes *1*, *2*, *5*, and *6*) and Syk (lanes *3*, *4*, 7, and *8*) were immunoprecipitated from lysates prepared from the SCB.29 (lanes *1–4*) and SCαβ (lanes *5–10*) pre–T cell lines before (lanes *1*, *3*, *5*, 7, and *9*) and after receptor ligation (lanes *2*, *4*, *6*, *8*, and *10*). The precipitates were subjected to in vitro kinase assays, resolved by SDS-PAGE, transferred to PVDF, KOH treated, and analyzed by autoradiography. Precipitates formed with NRS were included as controls.

TCR complex with an anti-CD3ϵ mAb revealed a marked increase in ZAP-70 phosphorylation as well as the phosphorylation and association of the p21 and p23 forms of phospho-ζ with ZAP-70. These findings were confirmed after the analysis of TCR-ζ immunoprecipitates. Thus, cross-linking the pre-TCR complex resulted in the phosphorylation of the p21 and p23 forms of phospho-ζ as well as the phosphorylation and association of ZAP-70 (Fig. 2 *C*, lanes *5* and *6*). We have also determined that the Syk PTK becomes inducibly tyrosine phosphorylated and associates with phospho-ζ in the pre-TCR–expressing cells (data not shown). These results directly demonstrate that the TCR-ζ subunit is functionally coupled to the CD3ϵ chain in spite of the fact that TCR-ζ cannot be detected in pre-TCR immunoprecipitates. It is unlikely that cross-linking CD3ϵ in the pre-TCR complex contributes to a nonspecific phosphorylation of TCR-ζ, because previous studies with CD8/ζ chimera–expressing cells have shown that chimera cross-linking does not lead to TCR-ζ phosphorylation, and TCR cross-linking fails to influence the phosphorylation status of the chimera (27). Similar findings were also observed for the association of ZAP-70 with the TCR and/or the CD8/ζ chimera (28).

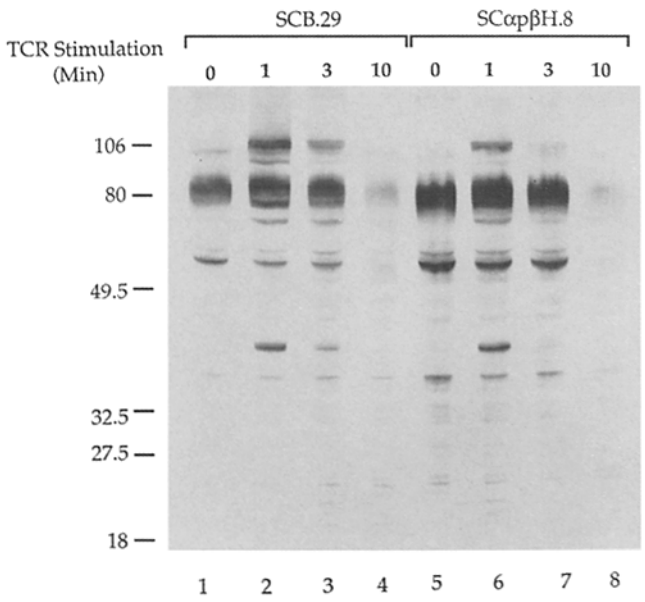

Figure 4. Induction of tyrosine phosphoproteins in pre-TCR and TCR-α/β–expressing pre–T cell lines. The SCB.29 and SCαβ cells were stimulated with an anti-CD3ϵ mAb (10 μg/ml) for the indicated times at 37°C and lysed, and the lysates were resolved by electrophoresis and analyzed by Western blotting with antiphosphotyrosine mAbs.

We have also examined the changes in ZAP-70 and Syk PTK activity after TCR ligation by precipitating ZAP-70 or Syk and performing in vitro kinase assays (Fig. 3). ZAP-70 exhibited a small increase in kinase activity, as assessed by autophosphorylation after receptor engagement (Fig. 3, lanes *2* and *6*). The increase in ZAP-70 autophosphorylation was similar for both the pre-TCR and TCR-α/β–expressing cell lines (lanes *2* and *6* versus *1* and *5*). In addition, Syk also appeared to increase its kinase activity in the pre-TCR cells (lane *4* versus lane *3*). However, Syk PTK activation was only occasionally detected in the TCR-α/β–expressing cells, perhaps because of the lower expression of this receptor (lanes 7 and *8*). Although the increases in autophosphorylation detected for both kinases most likely reflect an increase in ZAP-70 or Syk activity, additional PTKs such as Lck may also contribute to the demonstrated changes after TCR engagement (29). In summary, the data demonstrate that the pre-TCR complex is competent at transducing intracellular signals after receptor ligation, leading to the activation of both the ZAP-70 and Syk PTKs.

The physiological relevance of a pre-TCR complex maintaining a very weak biochemical association with TCR-ζ remains to be established. In fact, we cannot rule out the possibility that the TCR-ζ chain simply dissociates from the pre-TCR under the in vitro lysis conditions used. For example, the human TCR-ζ chain dissociates from human CD3ϵ under the Triton X-100 lysis conditions we have used (data not shown). Alternatively, pre-TCR ligation could lead to TCR-ζ phosphorylation and ZAP-70 recruit-

ment, forming a complex that dissociates from the pre-TCR complex and maintains a prolonged signaling module influencing distinct downstream pathways. However, we have been unable to detect substantial differences in the kinetics of TCR-ζ phosphorylation/dephosphorylation and/or ZAP-70 association with phospho-ζ in comparative studies between the pre-TCR and TCR-α/β–expressing cells (unpublished observations). We have also examined the kinetics and patterns of substrate phosphorylation after TCR ligation in the SCB.29 and SCαβ cells. As illustrated in Fig. 4, the patterns of induced phosphoproteins are qualitatively similar between the pre-TCR– and TCR-α/β–expressing cells over the 10-min time course analyzed. Thus, there are no obvious differences in signal transduction processes between a TCR-α/β or pre-TCR complex expressed in the immature Scid-derived cell lines using the assays described in this paper. However, we cannot exclude the possibility that these receptors mediate distinct signals when expressed on the surface of thymocytes at different stages of development.

Our results indicate that the TCR-ζ chain contributes to signaling mediated through the pre-TCR complex. Since the expansion of the $CD4^+CD8^+$ thymocytes is regulated by the pre-TCR complex (unpublished results), TCR-ζ functions are likely to be required for these events. This is consistent with the observation that $CD4^+CD8^+$ thymocytes are present in TCR-ζ–deficient mice, but their numbers are reduced 10–20-fold relative to normal mice (18). Moreover, the expression of TCR-ζ transgenes with increasing numbers of ITAMs in the ζ-deficient mice promotes increases in total thymocyte numbers (18).

In summary, our studies demonstrate that the pre-TCR complex is functionally coupled to the TCR-ζ chain, and pre-TCR engagement leads to the tyrosine phosphorylation of TCR-ζ and CD3ε, and to association and phosphorylation of the ZAP-70 and Syk PTKs. The questions of whether the weak biochemical association between the pre-TCR complex and TCR-ζ has a physiologically relevant function for pre-TCR cell development and whether specific signaling molecules with unique signaling functions associate with the pTα chain cytoplasmic domain require further elucidation.

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