Subunit Composition of Pre–T Cell Receptor Complexes Expressed by Primary Thymocytes: CD3δ Is Physically Associated but Not Functionally Required

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Summary

Maturation of immature $CD4^-CD8^-$ (DN) thymocytes to the $CD4^+CD8^+$ (DP) stage of development is driven by signals transduced through a pre–T cell receptor (TCR) complex, whose hallmark is a novel subunit termed pre-Tα (pTα). However, the precise role of pre-TCRs in mediating the DN to DP transition remains unclear. Moreover, progress in understanding pre-TCR function has been hampered thus far because previous attempts to demonstrate expression of pTα-containing pre-TCRs on the surface of normal thymocytes have been unsuccessful. In this report, we demonstrate for the first time that pTα-containing pre-TCR complexes are expressed at low levels on the surface of primary thymocytes and that these pre-TCR complexes comprise a disulfide-linked pTα–TCR-β heterodimer associated not only with CD3-γ and -ε, as previously reported, but also with ζ and δ. Interestingly, while CD3-δ is associated with the pre-TCR complex, it is not required for pre-TCR function, as evidenced by the generation of normal numbers of DP thymocytes in CD3-δ–deficient mice. The fact that any of the signaling components of the pre-TCR are dispensable for pre-TCR function is indeed surprising, given that few pre-TCR complexes are actually expressed on the surface of primary thymocytes in vivo. Thus, pre-TCRs do not require the full array of TCR-associated signaling subunits (γ, δ, ε, and ζ), possibly because pTα itself possesses signaling capabilities.

Maturation of immature thymocytes from the $CD4^-CD8^-$ (DN)[1] to the $CD4^+CD8^+$ (DP) stage of development requires productive rearrangement of the gene segments encoding TCR-β (1–3). The fidelity with which TCR-β gene rearrangement has occurred is thought to be assessed using a surrogate receptor complex termed the pre-TCR, which is defined by a novel 33-kD pre-Tα (pTα) subunit (4). Upon productive rearrangement of TCR-β, the pre-TCR complex transduces signals through CD3 (and possibly also pTα) that direct allelic exclusion at the TCR-β locus and promote maturation of DN thymocytes to the DP stage (5); however, the molecular details of how pre-TCR complexes discriminate between aberrant and productive β gene rearrangements remain unclear.

Progress towards a deeper understanding of pre-TCR complex function is hampered by the absence of a precise description of pre-TCR subunit composition. At present, the pre-TCR complex is thought to consist of a disulfide-linked pTα–TCR-β (pTα–β) heterodimer associated with an inadequately defined complement of signaling molecules (5). Indirect support for the presence of particular TCR–CD3 chains within the pre-TCR complex comes from gene-targeted mice, which reveal that the DN to DP transition is attenuated by elimination of TCR-β, pTα, or TCR-ζ alone, or by simultaneous elimination of CD3-γ/δ/ε (2, 6–8). Direct biochemical analysis of pre-TCR composition has yielded only incomplete and contradictory results, particularly regarding the CD3-δ and TCR-ζ subunits, possibly because of the idiosyncrasies of thymic lymphoma lines used in these analyses (4, 9–11). These discrepancies could be resolved, if it were possible to analyze pre-TCR structure using normal primary thymocytes; however, thus far, attempts to do so have been unsuccessful (4, 12).

This study comprises a rigorous biochemical analysis of surface TCR expression that clarifies these issues. We pro-

[1]*Abbreviations used in this paper:* 2D NR×R, two-dimensional nonreducing × reducing; CD3-δ^0, CD3-δ–deficient; DN, $CD4^-CD8^-$ double negative; DP, $CD4^+CD8^+$ double positive; HRP-Av, horseradish peroxidase–conjugated streptavidin; pTα, pre-Tα; pTα–β, pTα–TCR-β; TCR-α^0, TCR-α–deficient.

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vide the first demonstration that pTα-containing pre-TCR complexes are actually expressed at low levels on the surface of normal thymocytes in vivo; moreover, we have defined their subunit composition. They consist of a disulfide-linked pTα–β heterodimer associated not only with CD3-γ/ϵ as was previously thought, but also with TCR-ζ and CD3-δ. In addition, we demonstrate that pre-TCR function is not attenuated in CD3-δ–deficient (CD3-δ^0) mice (13). Thus, while CD3-δ is a pre-TCR component, it is not required for pre-TCR function.

Materials and Methods

Animals. Mice lacking TCR-α expression (TCR-α^0) due to gene targeting (2, 14) were obtained from The Jackson Laboratory (Bar Harbor, ME) and then maintained in our colony. CD3-δ^0 mice were generated as previously described (13). Production of TCR-α^0CD3-δ^0, and TCR-α^0CD3-δ^+ mice was achieved by intercrossing the F1 progeny of a TCR-α^0 × CD3-δ^0 mating and then screening F2 mice by Southern blot as previously described (15).

Cell Lines and Antibodies. The DP α/β-TCR$^+$ thymic lymphoma VL3-3M2 (obtained from Dr. C. Guidos, Hospital for Sick Children, Toronto, Canada) and a spontaneous thymic lymphoma from SCID mice, SL-12 (TCR$^-$; reference 16) were maintained in RPMI supplemented as previously described (17). The following mAbs were used: anti–TCR-β (H57-597); anti–CD3-γ/ϵ (7D6); and anti–TCR-α (H28-710). The following polyclonal rabbit Abs were used: anti–TCR-ζ (551; reference 17); anti-CD3-δ (R9; gift of Dr. Lawrence Samelson, National Institutes of Health [NIH], Bethesda, MD); and anti-pTα. The anti-pTα Ab was raised against a GST (glutathione-S-transferase) fusion protein encompassing the cytoplasmic domain of pTα as previously described (17).

Plasmid Construction. The pTα cDNA was cloned by performing reverse transcriptase–PCR on total RNA from day 15 fetal thymocytes (gift of Dr. Paul Love, National Institute of Child Health and Human Development [NICHD], Bethesda, MD) using standard methodology (15). First, strand cDNA was synthesized using the Superscript Preamplification System (GIBCO BRL, Bethesda, MD), and then the pTα cDNA was amplified by PCR (MJ Research, Inc., Watertown, MA) using primers flanking the coding region. The resultant fragment was cloned into pCR2.1 (Invitrogen, San Diego, CA) and verified by automated sequencing in our facility. The pTα cytoplasmic tail–GST fusion protein was generated by PCR amplification of the pTα cDNA using primers that appended SmaI and EcoRI linkers onto the cytoplasmic tail fragment. The fragment was ligated into pCR2.1, excised using SmaI and EcoRI, and directionally cloned in frame into pGEX-2T (Pharmacia Biotech Inc., Piscataway, NJ). After transformation into BL21pLysS cells, expression of the pTα cytoplasmic tail–GST (glutathione-S-transferase) fusion protein was induced, after which it was purified from bacterial extracts using glutathione-Sepharose (Pharmacia Biotech Inc.) according to the manufacturer's recommendations (18).

A 1.1-kb XhoI-XbaI cDNA fragment encoding the TCR-β subunit of the 2B4 TCR (pCDMB–2B4-β; provided by Dr. Juan Bonifacino, NICHD, Bethesda, MD) was subcloned into the pXS expression vector (gift of Dr. Juan Bonifacino) using standard methodology (15).

Transfection. pXS–2B4-β and the neomycin resistance plasmid pFneo were linearized with HindIII and EcoRI, respectively, and then transfected into SL-12 SCID thymic lymphoma cells by electroporation (300 V, 400 μFa) using a BTX electroporation unit (BTX, Inc., San Diego, CA). Clones that were resistant to G418 (Boehringer Mannheim, Indianapolis, IN) were analyzed for TCR-β expression by flow cytometry.

Biotin Labeling, Immunoprecipitation, and Electrophoresis. Biotin surface labeling, after which cell viability was consistently >98%, was performed as previously described (17, 18). Cell lysis, immunoprecipitation, and electrophoresis were as previously described (17, 18). Electrophoretically separated surface-biotinylated proteins were transferred to membranes and visualized with horseradish peroxidase–conjugated streptavidin (HRP-Av; Southern Biotechnology Associates, Inc., Birmingham, AL) as previously described (17).

Recapture Assay. Recapture assays were performed as previously described (17) using the anti-pTα Ab. The resultant immune complexes were resolved either on two- or one-dimensional nonreducing SDS-PAGE gels, after which the surface-labeled proteins were visualized as above.

Flow Cytometry. Flow cytometry was performed as previously described (18).

Results

Expression of pTα-containing Pre-TCR Complexes on the Surface of Primary Thymocytes. It is well established that immature thymocytes express surface TCR complexes containing TCR-β without TCR-α; however, it remains unclear whether these complexes comprise TCR-β homodimers or, alternatively, heterodimers of TCR-β and pTα, the hallmark of the pre-TCR complex (4, 12, 19). This issue remains unresolved because previous attempts to demonstrate expression of pTα-containing pre-TCR complexes on the surface of primary thymocytes have been unsuccessful (4, 12, 19). Consequently, we wished to determine if pTα-containing pre-TCR complexes were actually expressed on the surface of primary thymocytes. Thymocytes from TCR-α^0 mice were used because they lack α/β-TCR complexes which might otherwise complicate analysis of pre-TCR structure, yet they still exhibit normal pre-TCR function (2, 14). Detergent extracts of surface biotin–labeled

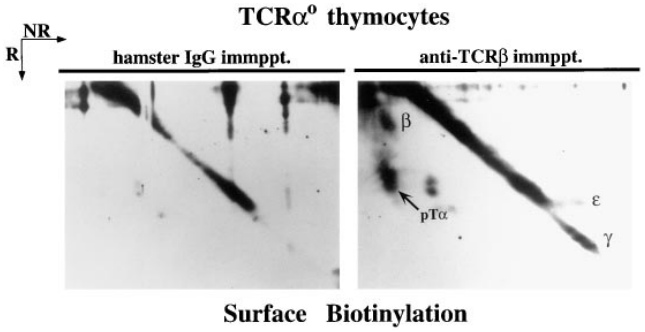
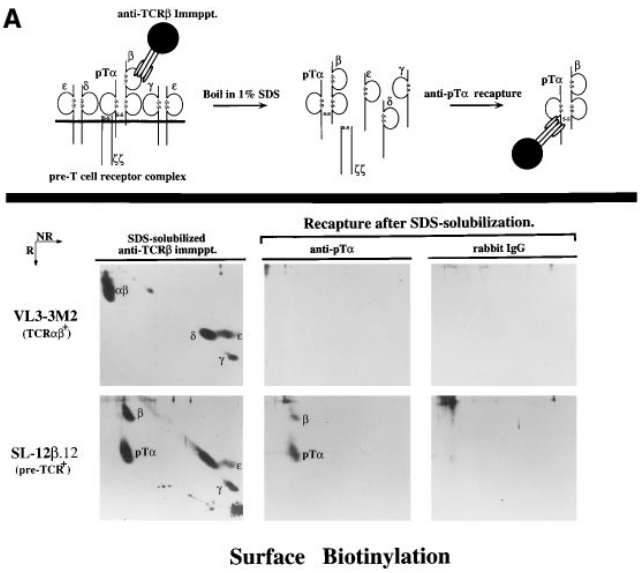


Figure 1. Expression of pTα-containing pre-TCR complexes on the surface of primary thymocytes. Surface biotin–labeled thymocytes from TCR-α^0 mice were solubilized in Brij 96, immunoprecipitated with anti–TCR-β Ab (H57-597; *right*), or control hamster IgG (*left*) and resolved on 2D NR×R SDS-PAGE gels. Surface-labeled proteins were visualized by HRP-Av and chemiluminescence. Migration positions of individual subunits are indicated.



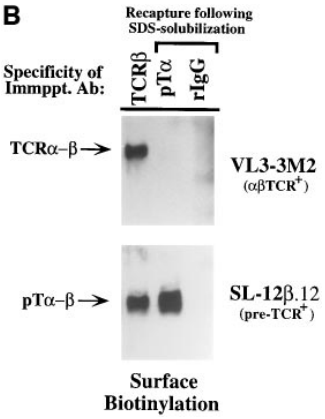


Figure 2. pTα–β heterodimers are specifically recaptured by Abs reactive with the cytoplasmic domain of pTα. (*A*) Recapture assay. Recapture assay is schematized in the top panel. (*Bottom*) Brij 96 detergent extracts of surface-labeled VL3-3M2 and SL-12β.12 cells were immunoprecipitated with anti–TCR-β. After elution by boiling in SDS, the anti–TCR-β immune complexes were quenched with NP-40 and split into three equal parts which were either: (*a*) held on ice; (*b*) immunoprecipitated with anti-pTα Ab; or (*c*) immunoprecipitated with control rabbit IgG. The samples were then resolved on 2D NR×R SDS-PAGE gels, after which the surface-labeled proteins were visualized with HRP-Av and chemiluminescence. (*B*) Anti-pTα Ab specifically recaptured pTα–β heterodimers from detergent extracts of surface-labeled pre-TCR$^+$ (SL-12β.12) but not from α/β-TCR$^+$ (VL3-3M2) cells. A recapture assay was performed as above except that the samples were resolved on one-dimensional SDS-PAGE under nonreducing conditions.

thymocytes were immunoprecipitated either with control hamster IgG or with an anti–TCR-β mAb after which the resultant immune complexes were resolved by two-dimensional nonreducing × reducing (2D NR×R) SDS-PAGE (Fig. 1). The anti–TCR-β mAb immunoprecipitation contained associated CD3-γ/ε heterodimers as well as disulfide-linked pTα–β heterodimers. Curiously, neither surface-labeled TCR-ζ nor CD3-δ was visible. Thus, primary thymocytes do indeed express surface pre-TCR complexes in which pTα–β and CD3-γ/ε heterodimers are evident.

The apparent absence of CD3-δ and TCR-ζ from pre-TCR complexes does not exclude them as potential pre-TCR components, as subunit visibility in this assay is dependent upon accessibility during surface labeling. Accordingly, proteins that did not label efficiently would be missed, providing an inaccurate view of pre-TCR subunit composition. In particular, surface-labeled TCR-ζ molecules were conspicuously absent from α/β-TCR complexes expressed by the VL3-3M2 thymic lymphoma (Fig. 2 *A*), despite the fact that these α/β-TCR complexes do contain TCR-ζ (data not shown). Likewise, our inability to identify labeled CD3-δ subunits in the pre-TCRs expressed by primary thymocytes may result from inefficient biotin-labeling of CD3-δ and/or from comigration with a contaminating protein that obscures CD3-δ.

Recapture Assay for Analyzing Pre-TCR Composition. To circumvent the limitations outlined above, we used a coimmunoprecipitation strategy to rigorously determine the subunit composition of the pre-TCR complex. A subunit was deemed part of the pre-TCR complex if Abs reactive with that subunit coprecipitated surface-labeled pTα, the hallmark of the pre-TCR complex. Coimmunoprecipitated pTα molecules were identified using the recapture assay, which occurs in three phases (Fig. 2 *A*, *top*): (*a*) Ab reactive with TCR subunits were used to coimmunoprecipitate pTα molecules (if associated) from cell extracts made with detergents selected for their ability to maintain the noncovalent interactions between subunits; (*b*) noncovalent interactions were disrupted by boiling the immune complex in 1% SDS; and (*c*) coimmunoprecipitated pTα–β heterodimers were identified by using anti-pTα Abs to recapture them from among the SDS-solubilized proteins. To verify that the anti-pTα Abs specifically recaptured pTα–β heterodimers, the recapture assay was performed on anti–TCR-β immunoprecipitates from both α/β-TCR–expressing (VL3-3M2) and pre-TCR–expressing (SL-12β.12) thymic lymphoma cells (Fig. 2 *A*, *bottom*). Parallel anti–TCR-β immunoprecipitates were resolved on 2D NR×R SDS-PAGE gels to reveal the protein composition of the original immunoprecipitation. In the anti–TCR-β immunoprecipitations from pre-TCR$^+$ cells, heterodimers of pTα–β and CD3-γ/ε were evident, whereas in those from α/β-TCR$^+$ cells, we observed heterodimers of TCR-α and -β in association with CD3-γ/δ/ε, but not pTα (Fig. 2 *A*, *bottom left*). The anti-pTα Ab recaptured pTα–β heterodimers from pre-TCR–expressing SL-12β.12 cells, but not from cells expressing only α/β-TCR complexes (VL3-3M2; Fig. 2 *A*, *bottom center*), demonstrating that the recapture assay specifically identifies pTα–β heterodimers. This was also evident in a similar experiment in which the samples were resolved on one-dimensional gels in which the interchain disulfide bonds between pTα and TCR-β were maintained (Fig. 2 *B*).

Composition of Pre-TCR Complexes Expressed on the Surface of Primary Thymocytes. Having established that the recapture assay was specific, we next wished to evaluate subunit composition of pre-TCR complexes expressed on the surface of primary thymocytes. Abs reactive with TCR-β, CD3-γ/ε, TCR-ζ, and CD3-δ coprecipitated pTα–β heterodimers from detergent extracts of surface-labeled thymocytes (Fig. 3 *A*). Thus, according to the above definition, our analysis demonstrates that the pre-TCR complexes expressed by primary thymocytes in vivo comprise pTα–β heterodimers associated with CD3-γ/δ/ε, and TCR-ζ.

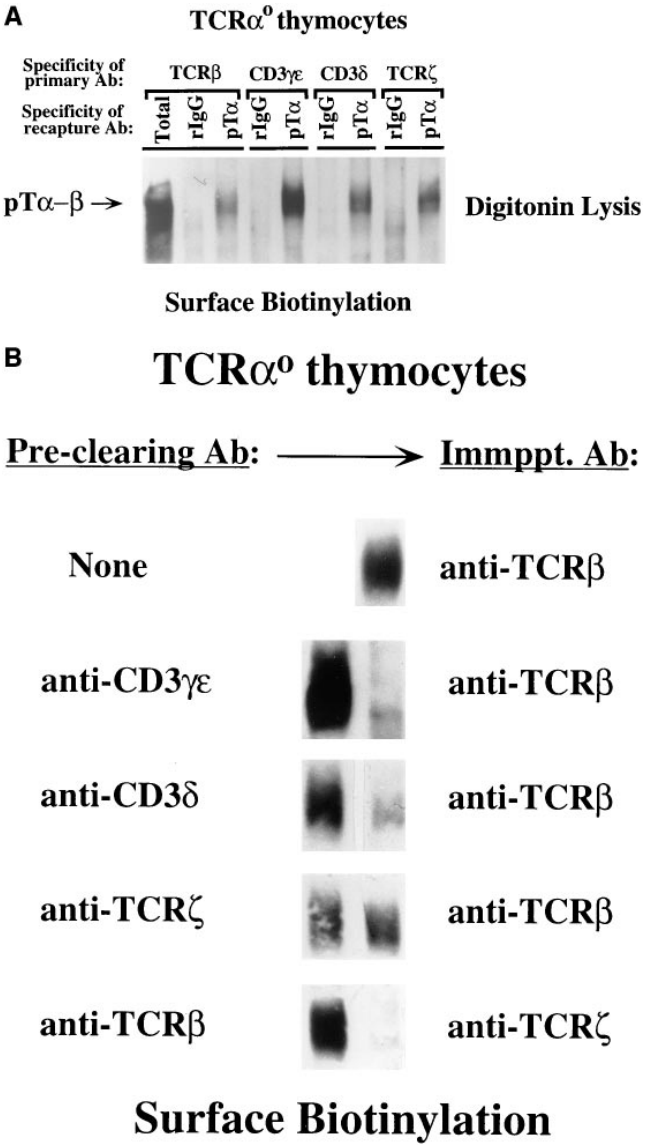


Figure 3. Subunit composition of pre-TCR complexes expressed by primary explanted thymocytes. (*A*) Digitonin extracts of biotin–labeled TCR-α^0 thymocytes were immunoprecipitated using Abs reactive with the following subunits: TCR-β (H57-597), CD3-γ/ϵ (7D6), CD3-δ (R9) or TCR-ζ (551). SDS eluates of the resultant immune complexes were either resolved directly on SDS-PAGE gels under nonreducing conditions (*Total*) or after neutralization and immunoprecipitation with either anti-pTα (*pTα*) or control rabbit IgG (*rIgG*). Control anti–TCR-α Ab did not coprecipitate pTα–β heterodimers from TCR-α^0 thymocytes (data not shown). (*B*) CD3-γ/δ/ϵ and TCR-ζ are all associated with pTα–β heterodimers within the same pre-TCR complex. Digitonin extracts of biotin–labeled TCR-α^0 thymocytes were either immunoprecipitated directly with anti–TCR-β or after the detergent extracts had been depleted of CD3-δ, -γ/ϵ, or -ζ with Abs reactive with those proteins. Surface-labeled proteins were visualized by HRP-Av and chemiluminescence.

To determine if CD3-γ/ϵ, CD3-δ, and TCR-ζ were all associated with pTα–β within the same complex, we performed a series of sequential immunoprecipitations to see if all of the pTα–β heterodimers could be depleted from the detergent extracts by exhaustive preclearing using Abs reactive with those proteins (Fig. 3 *B*). Indeed, virtually all of the pTα–β heterodimers expressed on the surface of TCR-α^0 thymocytes could be precleared using Abs reactive with either CD3-γ/ϵ or -δ. In contrast, anti–TCR-ζ Ab was able to preclear only ~50% of the pTα–β heterodimers, indicating that either ζ is associated with only half of the pre-TCR complexes expressed by primary thymocytes or, alternatively, that ζ association with the pre-TCR is weak. We favor the latter interpretation because ζ association with the pre-TCR complex, unlike that of the other pre-TCR subunits, was easily disrupted by solubilization in harsh detergents (data not shown). Taken together, these data demonstrate that all pre-TCR complexes expressed on the surface of primary thymocytes in vivo contain pTα–β heterodimers associated with CD3-γ/δ/ϵ, and of these at least half are also associated with TCR-ζ.

Loss of CD3-δ Does Not Affect Development of Thymocytes from the DN to the DP Stage. Since we had demonstrated CD3-δ was a component of the pre-TCR complex, it was important to determine if δ were critical to pre-TCR function. Thus, we analyzed TCR-α^0CD3-δ^0 mice to determine if the loss of the CD3-δ signaling component affected pre-TCR expression as well as two manifestations of pre-TCR function, thymic cellularity and maturation of DN thymocytes to the DP stage (5, 6). Thymocytes from TCR-α^0CD3-δ^0 mice expressed surface pre-TCRs comprising pTα–β heterodimers associated with CD3-γ/ϵ and TCR-ζ, indicating that CD3-δ deficiency does not prevent assembly and surface expression of the remaining pre-TCR subunits (Fig. 4, *A* and *B*, and data not shown). Likewise, δ deficiency did not attenuate pre-TCR function. Flow cytometric analysis of thymocytes from TCR-α^0CD3-δ^+ and TCR-α^0CD3-δ^0 mice revealed that each contained 96% DP thymocytes (Fig. 4 *B*). Furthermore, total thymic cellularity in TCR-α^0CD3-δ^0 mice was equivalent to that in TCR-α^0CD3-δ^+ mice (2.80×10^8 versus 3.31×10^8, respectively; $P < 0.01$). Thus, the absence of CD3-δ from the pre-TCR complex does not adversely affect the progression of immature thymocytes from the DN to the DP stage of development, demonstrating that CD3-δ is not necessary for pre-TCR function.

Discussion

Progress in understanding the molecular details of how the pre-TCR regulates early thymocyte development has been hampered by the absence of a precise description of pre-TCR subunit composition. This report addresses this problem. We provide the first demonstration that primary thymocytes express pTα-containing pre-TCR complexes on the cell surface and we have elucidated their subunit composition. They consist of pTα–β heterodimers associated not only with CD3-γ/ϵ as was previously thought, but also with TCR-ζ and CD3-δ subunits. Finally, we found that despite being a component of the pre-TCR, CD3-δ is dispensable for the biological role of the pre-TCR complex.

Before our study, it was unclear whether the TCR-β

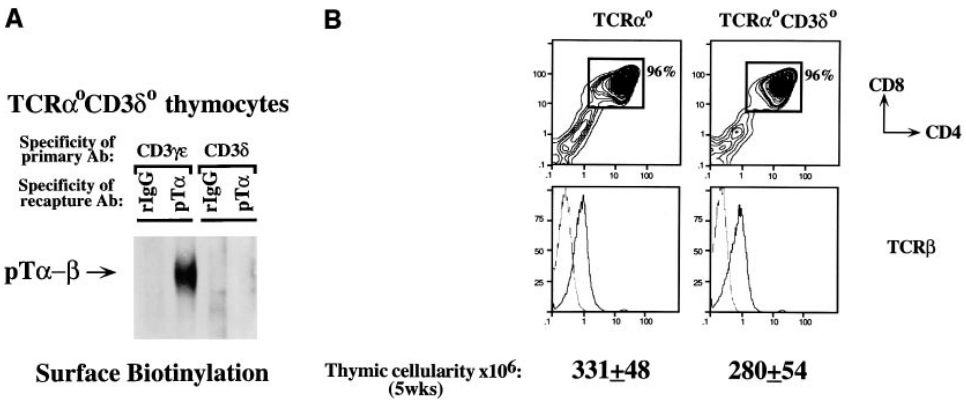


Figure 4. The CD3-δ deficiency does not attenuate the DN to the DP transition. (*A*) The absence of CD3-δ does not affect pre-TCR expression. Digitonin extracts of surface biotin–labeled TCR-α^0CD3-δ^0 thymocytes were immunoprecipitated with anti–CD3-γ/ε or anti–CD3-δ Abs after which the resultant immune complexes were analyzed by recapture assay. Immune complexes were resolved by one-dimensional SDS-PAGE under nonreducing conditions. The absence of any recaptured pTα–β heterodimers in the anti–CD3-δ immunoprecipitations demonstrates the specificity of the anti–CD3-δ Ab. (*B*) Thymocytes from both TCR-α^0CD3-δ^+ and TCR-α^0CD3-δ^0 mice have the same percentage of $CD4^+CD8^+$ cells (*top*) and express similar levels of TCR-β on the cell surface (*bottom*; *thick line*), as measured by flow cytometry with fluorochrome conjugated mAbs. The thin line represents control staining with anti–human CD3-ε Ab. The mean number of cells per thymus for each strain is indicated at the bottom of the figure.

complexes (without TCR-α) that were expressed on developing thymocytes contained TCR-β homodimers or, alternatively, disulfide-linked pTα–β heterodimers, the hallmark of the pre-TCR complex (4, 12, 19). Moreover, because of previous failures to demonstrate surface expression of pTα in vivo, it was proposed that pre-TCR complexes evaluate TCR-β protein structure not through interactions with an extracellular ligand at the cell surface, but rather from the cell interior (5), possibly during subunit assembly within the endoplasmic reticulum. Consistent with this viewpoint, a TCR-β transgene lacking the variable domain is able to allelically exclude endogenous TCR-β rearrangement and promote the DN to DP transition, both hallmarks of pre-TCR function (20). Moreover, analysis of the efficiency with which TCR-β transgenic DN precursors differentiate to the DP stage suggests that this transition is not constrained by a limiting number of intrathymic "niches" or extracellular ligands, as is true of the antigen-driven selection events that promote maturation of DP thymocytes to the $CD4^+$ or $CD8^+$ stage (21). Thus, if pre-TCR complexes do evaluate the fidelity of TCR-β gene rearrangement by interacting with an extracellular ligand, then that ligand does not absolutely require the TCR-β variable domain, nor is it present in limiting quantities. While we provide the first compelling demonstration that primary thymocytes express pTα-containing pre-TCR complexes on the cell surface (Figs. 1 and 3), this does not rule out the possibility that pre-TCR complexes might function from the cell interior.

Previous analyses of pre-TCR structure were consistent in indicating that pre-TCRs contained pTα, TCR-β, CD3-γ, and CD3-ε; however, there were conflicting data regarding CD3-δ and TCR-ζ (4, 9–11). CD3-δ was found to be a component of pre-TCR complexes in some tumor lines, but not in others (9, 10). Furthermore, TCR-ζ association with the pre-TCR has been implicated by functional criteria but not by physical association (11). These discrepancies might result either from idiosyncrasies of the lymphoma cell lines used or from the experimental conditions (9–11). In particular, the detergent used in cell lysis can markedly affect association of individual subunits with the pre-TCR. We found that association of TCR-ζ with the pre-TCR complex could be more easily disrupted by lysis in harsh detergents than that of CD3-γ/δ/ε (data not shown). This was not true for ζ association with the α/β-TCR complex (data not shown). Finally, while we have elucidated the subunit composition of pre-TCRs expressed by TCR-α^0 thymocytes, this population consists primarily of DP thymocytes and so it remains possible that distinct subpopulations of DN thymocytes might express alternative forms of the pre-TCR complex. Experiments are currently in progress to investigate this possibility.

The ability of TCR and pre-TCR complexes to transduce signals resides in their invariant CD3-γ/δ/ε and TCR-ζ subunits. While both receptors carry the same array (γ, δ, ε, and ζ), the requirements of these receptors for individual signaling subunits differ (Fig. 5), raising the fundamental question of whether the different subunits subserve redundant or unique roles in receptor function. If CD3-γ/δ/ε and TCR-ζ subunits are redundant and function to amplify signals, then it is surprising that the pre-TCR complex can tolerate the loss of CD3-δ, given that surface expression levels of the pre-TCR complex are so low (Figs. 1 and 4). In that regard, the pre-TCR may be able to tolerate loss of CD3-δ because pre-TCR signals need not be as quantitatively intense or because pre-TCRs have a lower signaling threshold (than α/β-TCR complexes). In support of the latter possibility, pre-TCR complexes function before expression of surface molecules that can be inhibitory, such as CD4, which we have shown can decrease signaling competence of the α/β-TCR on DP thymocytes by sequestering $p56^{lck}$ protein tyrosine kinase (22). It is also possible that the individual CD3-γ/δ/ε and TCR-ζ signaling subunits perform unique functions. Consistent with this hypothesis, it has been shown that immunoreceptor tyrosine-based activation motifs of different signaling subunits are able to interact with different cytoplasmic signaling effector molecules and induce phosphorylation of different substrates (23). Finally, it is possible that pre-TCR complexes can tolerate loss of CD3-δ because in the ab-

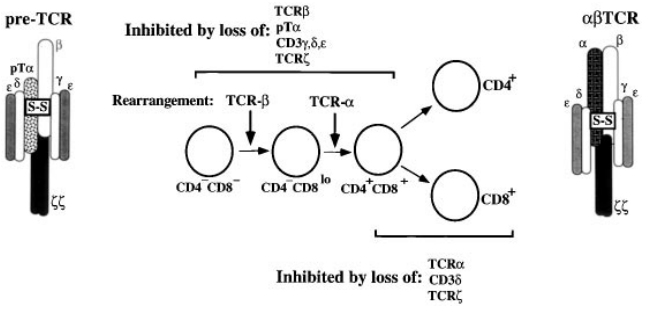


Figure 5. Schematic of the role of the pre-TCR in thymocyte development. After β gene rearrangement, immature thymocytes express surface pre-TCR complexes comprising pTα–β heterodimers associated with CD3-γ/δ/ϵ and TCR-ζ signaling subunits. Pre-TCR complexes are thought to regulate development of thymocytes from the DN to the DP stage by evaluating the status of the TCR-β protein product after β gene rearrangement. The molecular interactions underlying this evaluation process are currently unclear; however, development is blocked by elimination of pTα, TCR-ζ or CD3-γ/δ/ϵ, but not CD3-δ. Curiously, elimination of CD3-δ does impair the ability of the α/β-TCR to signal maturation of DP thymocytes to the $CD4^+$ or $CD8^+$ stage. Thus, while both pre-TCR and TCR complexes possess the same array of signaling subunits, they exhibit differential dependence on the individual components.

sence of CD3-δ the pTα subunit itself is also able to function as a signaling subunit. Murine pTα has a cytoplasmic tail of ~30 amino acids which contains consensus motifs for phosphorylation by protein kinase C and for docking of SH3 domain containing proteins (12); however, the functional importance of these motifs is unclear as there is little sequence conservation between the cytoplasmic tails of the murine and human pTα homologues (5). Recently, the role of the cytoplasmic tail of pTα was tested by reconstitution of pTα-deficient mice with a pTα transgene lacking the cytoplasmic tail (24). While tailless pTα compensated for pTα deficiency, it did so only partially, leaving open the possibility that the cytoplasmic tail of pTα does function as a signaling domain within the pre-TCR complex and might underlie the pre-TCR's ability to tolerate loss of the CD3-δ subunit. In that regard it would be informative to analyze the function of pre-TCR complexes in CD3-δ^0 thymocytes expressing tailless pTα molecules.

In summary, this study not only provides the first demonstration that pTα-containing pre-TCRs are expressed on the surface of primary thymocytes in vivo, but also provides a precise description of pre-TCR subunit composition. Contrary to previous reports, we found that pre-TCR complexes contained the CD3-δ subunit, but, importantly, did not require CD3-δ to fulfill their biological role in regulating early thymocyte development. Curiously, loss of CD3-δ does interfere with function of the α/β-TCR complex (13), illustrating that while both receptors possess the same array of TCR signaling components (γ, δ, ϵ, and ζ), their dependence on individual subunits differs. A deeper understanding of how individual signaling subunits function in the pre-TCR and α/β-TCR complexes must await the generation of new strains of transgenic mice bearing signaling subunits with mutated immunoreceptor tyrosine-based activation motifs.

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