An Endoplasmic Reticulum Retention Function for the Cytoplasmic Tail of the Human Pre–T Cell Receptor (TCR) α Chain: Potential Role in the Regulation of Cell Surface pre-TCR Expression Levels

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Abstract

The pre-T cell receptor (TCR), which consists of a TCR-β chain paired with pre–TCR-α (pTα) and associated with CD3/ζ components, is a critical regulator of T cell development. For unknown reasons, extremely low pre-TCR levels reach the plasma membrane of pre-T cells. By transfecting chimeric TCR-α–pTα proteins into pre-T and mature T cell lines, we show here that the low surface expression of the human pre-TCR is pTα chain dependent. Particularly, the cytoplasmic domain of pTα is sufficient to reduce surface expression of a conventional TCR-α/β to pre-TCR expression levels. Such reduced expression cannot be attributed to qualitative differences in the biochemical composition of the CD3/ζ modules associated with pre-TCR and TCR surface complexes. Rather, evidence is provided that the pTα cytoplasmic tail also causes a reduced surface expression of individual membrane molecules such as CD25 and CD4, which are shown to be retained in the endoplasmic reticulum (ER). Native pTα is also observed to be predominantly ER localized. Finally, sequential truncations along the pTα cytoplasmic domain revealed that removal of the COOH-terminal 48 residues is sufficient to release a CD4-pTα chimera from ER retention, and to restore native CD4 surface expression levels. As such a truncation in pTα also correlates with enhanced pre-TCR expression, the observed pTα ER retention function may contribute to the regulation of surface pre-TCR expression on pre-T cells.

Key words: human pre–T cell receptor • pTα cytoplasmic tail • surface expression • endoplasmic reticulum retention • CD3 complex

Introduction

Development of mature α/β T cells inside the thymus is controlled at two distinct check-points by the sequential expression of the pre-TCR, and the mature TCR-α/β (1–3). Early in T cell development, pre-T cells that succeed in productive TCR-β gene rearrangements express a functional TCR-β chain which pairs with the invariant pre–TCR-α (pTα)1 chain and associates with CD3 components to form the pre-TCR (4–8). Signaling through this pre-TCR complex triggers a process, known as TCR-β selection, which induces the cellular expansion and maturation of CD4+CD8− double negative (DN) pre-T cells into CD4+CD8+ double-positive (DP) thymocytes (2, 3, 9–11), and results in the induction of a high rate of TCR-α gene rearrangements (12). On productive TCR-α gene rearrangements and substitution of pTα by TCR-α/β, the TCR-α/β is expressed associated with CD3, and DP thymocytes can undergo a second step of selection, known as TCR-α/β selection, during which thymocytes are rescued from programmed cell death and induced to differentiate into conventional single positive (SP) thymocytes, upon binding to self-peptide–MHC complexes expressed on thymic stromal cells (3).

In contrast to TCR-α/β selection, current data support the view that TCR-β selection is independent of binding to an extracellular (EC) ligand (13, 14) and no evidence for
the existence of such a ligand has so far been provided. However, exit from the endoplasmic reticulum (ER) and expression at the cell surface is mandatory for the pre-TCR complex to exert its function (15), although rules controlling the assembly and intracellular transport of pre-TCR and TCR complexes may differ markedly, as the pre-TCR has remained elusive until recently, evidence has now been provided that, in the mouse, it has the same subunit composition as does the TCR-α/β complex, differing only in that the TCR-α subunit has been replaced by pTα and that ζ association has been significantly weakened relative to its association with the mature TCR-α/β (4, 7, 8, 18). Because ζ is required for efficient surface expression of the other subunits of the mature TCR-α/β including the CD3 molecules (19–24), its weak biochemical association with the pre-TCR could well result in a decreased stability of the receptor complex at the cell surface. An alternative possibility is that, as reported for the pre-BCR, surface pre-TCR expression is controlled by a retention mechanism that is not selective for the pre-TCR, but is inherent to the pre-T cell stage (1, 17). In this study, we have examined this issue by performing transfections of human chimeric TCR-α-pTα molecules comprising distinct domains of TCR-α and pTα into either pre-T or mature T cell lines, and provide evidence that low surface expression of the human pre-TCR is intrinsic to the pTα chain. Particularly, our results show that the cytoplasmic (Cyto) domain of pTα is sufficient to promote retention in the ER and to reduce surface expression levels of individual transmembrane (TM) proteins. The possibility that the pTα Cyto tail functions as an ER retention signal that contributes to the regulation of pre-TCR expression levels on pre-T cells is discussed.

Materials and Methods

Isolation of Thymocyte Subsets. Postnatal thymocytes isolated from thymus samples removed during corrective cardiac surgery of patients aged 1 mo to 3 yr were fractionated by centrifugation on Percoll (Amersham Pharmacia Biotech) density gradients as described (25), pre-TCR/CD3low pre-T cells were isolated from the large-sized cell fraction by immunomagnetic sorting (Dynal) as described (16).

Flow Cytometry. PE-labeled anti-CD3 (Leu-4-PE), biotin-conjugated anti-CD25 (7D4), and Cy5-PE-labeled anti-CD4 mAbs were obtained from Becton Dickinson, BD PharMingen, and Caltag Laboratories, respectively. The BMA031 mAb, raised against a monomorphic determinant of TCR-α/β (26) was provided by Dr. R. Kuryle (Behringwerke AG, Marburg, Germany), and the 6D6 mAb, against the human Vα12I, TCR-α domain (27), was the gift of Dr. M. Brenner (Brigham and Women’s Hospital, Boston, MA). Surface expression of the human pTα chain was determined by staining with a rabbit antiserum (ED-1) raised against a synthetic peptide present in the pTα EC domain (16). PE-labeled streptavidin and goat anti–mouse fluorescein-, PE-, or PE-Cy5–coupled F(ab’)2, Igs were purchased from Caltag Laboratories. Stained cells were analyzed in a flow cytometer (EPICS XL; Coulter Corp.) as described (25). For flow cytometry, COS cells were removed from culture 48 h after transfection with PBS containing 0.02% EDTA.

Generation of cDNA Constructs. Full-length cDNAs encoding the human pTα and the AV12S1 TCR α chain, respectively, were cloned into the BamHI site of the pcDNA3 plasmid vector (Invitrogen), as described previously (16). TCR-α-pTα chimeric constructs (see Fig. 2 A) comprising the Vα12S1 and ζα domains of the AV12S1 TCR α chain fused to pTα cDNA lacking the leader sequence (αI), or encoding a AV12S1 TCR α chain in which either both the TM and Cyto domains (αII) or exclusively the Cyto domain (αIII) of TCR-α have been replaced with homologous domains from pTα, were generated by PCR using TCR-α and pTα cDNAs as templates, and specific oligonucleotides. The sense 5’-ATG GAT CCT CTA GAT TTT TGC CAG CCT GTT G-3’ primer (with a BamHI site), corresponding to the NH2-terminal region of the AV12S1 TCR α chain (16), was used in combination with three distinct antisense primers: 5’-CCC GGA TCC TGC TGG TGC CTG TTC CTG TTC-3’ (with a BamHI site), 5’-CCG AAT TCG GTT TTG AAA GTT TAG GTT CG-3’ (with an EcoRI site), or 5’-CCG AAT TCC CAG CTT CAG GAT CAG ATT AAA-3’ (with an EcoRI site), which correspond to the COOH-terminal region of either the ζα, the EC, or the TM domains of the AV12S1 TCR α chain, respectively. PCR products were either BamHI or BamHI/EcoRI digested, and ligated into pcDNA3 plasmid containing, respectively, a BamHI/XhoI PCR fragment coding for pTα lacking the leader sequence, an EcoRI/XhoI PCR fragment encoding both the TM and Cyto pTα domains, or an EcoRI/ XhoI PCR fragment encoding the Cyto tail of pTα. Such pTα PCR products were amplified from pTα cDNA with the antisense 5’-CCG ATA AGT CAG AGT CAG GCA GCA GCT CCA GCC TGC AG-3’ primer, corresponding to the COOH-terminal region of the pTα Cyto domain, and containing an XhoI site, in combination with the sense primers 5’-CCC-GGA TCC ATA TGC TAC CCA CAG GTG TGG GC-3’, including a BamHI site, or 5’-CGG AAT TCT GTG GCT GGG GGT CCT GGC-3’, with an EcoRI site, or 5’-CCG AAT TCT TAC CTT CAG TCT CCT GTG CG-3’, including an EcoRI site, respectively.

Full-length cDNAs encoding murine CD25 (provided by Dr. L.R. Borlado, Centro Nacional de Biotecnología, Madrid, Spain) or human CD4 (28) were subcloned into the BamHI site of pcDNA3. CD25-pTα and CD4-pTα chimeric constructs, comprising the complete EC and TM domains of CD25 or CD4 fused to the pTα Cyto tail, were generated by PCR amplification. CD25 PCR products obtained with the sense 5’-ATG GAT CCT CCA AGA TGG AGC CAC GTC TGC TG-3’ primer and the antisense 5’-GGG AAT TCC CCA GGT GAG CCC GCT CAG G-3’ primer were digested with BamHI/EcoRI, and CD4 PCR products obtained with the sense 5’-AGA GAG AGA GAG AAG CTT TCG GCA AGG CCA CAA TGA AC-3’...
primer and the antisense 5'-GCC AAT GCC GAG GAT GCC TAG CCC AAT G-3' primer were digested with HindIII/EcoRI. Digested PCR products were independently ligated into pcDNA3 containing the EcoRI/XhoI PCR fragment encoding the pTα Cyto tail described above. Truncated CD4-pTα chimeras lacking either the COOH-terminal 22 (CD4-pTα22) or 48 (CD4-pTα48) residues of the pTα Cyto tail were generated essentially as described for CD4-pTα chimeric constructs, except that pTα PCR was performed with the sense primer corresponding to the NH2-terminal region of the pTα Cyto domain described above, in combination with the antisense 5'-AGA GAG AGA GAG GAT ATC TCA AGC CTT GAG AGA TCT TG-3' or 5'-AGA GAG AGA GAG GAT ATC TCA TAC TGG GCT CCC GGG CT'T C-3' primers, for CD4-pTα22 and CD4-pTα48, respectively. EcoRI/EcoRV-digested PCR products were then ligated into pcDNA3 containing the HindIII/EcoRI-digested CD4 product described above. These antisense primers were independently used with a previously described sense primer (16) corresponding to the NH2-terminal pTα region to generate truncated pTα proteins with identical deletions in the Cyto tail (pTα22 and pTα48). EcoRI/EcoRV-digested products were independently ligated into pcDNA3. Both cDNAs as well as full-length pTα and TCR-α cDNAs (16) were subsequently subcloned into the bicistronic expression vector pCIGFP. To construct the pCIGFP plasmid, a NotI cassette containing an internal ribosomal entry site (IRES) sequence followed by the enhanced green fluorescent protein (EGFP) cDNA was transferred from the pLZRS retroviral vector (29), provided by Dr. H. Spits (Netherlands Cancer Institute, Amsterdam, The Netherlands), into the NotI site in the pcDNA3 vector. Tailless pTα cDNA was generated by amplification with the sense primer described previously (16) in combination with the 5'-GGG GGA TGG GCT CCC GGG CTT C-3' antisense primer, BamHI digested, and ligated into the pCIGFP bicistronic plasmid. Each construct was sequenced directly in pcDNA3. The pTα-EGFP construct has been described previously (16).

Cell Lines and Transfection. The TCR-α-deficient JR3.11 mutant, derived from the mature human T cell line Jurkat (30; provided by Dr. B. Rubin, Centre National de la Recherche Scientifique, Toulouse, France), the human pre-T cell line SUP-T1 (5), and the murine lymphoid cell line BW, were grown in RPMI 1640 (Biowhittaker) supplemented with 10% FCS (GIBCO BRL). COS cells were grown in DMEM (Brenner) in combination with Cy5-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories). For confocal analysis, the pTα-EGFP SUP-T1 stable transfectants were adhered to Poly L-Lys (Sigma-Aldrich) precoated coverslips (5 x 10⁵ cells/coverslip). Coverslips were then washed in PBS, fixed with 2% paraformaldehyde in PBS for 10 min, permeabilized for 5 min with 0.05% Triton X-100 (Sigma-Aldrich), and blocked with 2% BSA/PBS. Cells were then consecutively stained with the anti–TCR-β F(ab')₂ rabbit antibody (provided by Dr. M. Brenner) in combination with Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories), and with a rabbit anti-CD4 antibody (Jackson ImmunoResearch Laboratories). For confocal microscopy, the pTα-EGFP SUP-T1 stable transfectants were adhered to Poly L-Lys (Sigma-Aldrich) precoated coverslips (5 x 10⁵ cells/coverslip). Coverslips were then washed in PBS, fixed with 2% paraformaldehyde in PBS for 10 min, permeabilized for 5 min with 0.05% Triton X-100 (Sigma-Aldrich), and blocked with 2% BSA/PBS. Cells were then consecutively stained with the anti–TCR-β F(ab')₂ rabbit antibody (provided by Dr. M. Brenner) in combination with Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Confo- cal microscopy was performed on a Radiance 2000 (Bio-Rad Laboratories) system coupled to an Axiovert S100TV inverted microscope (ZEISS). Fluorescein and EGFP, Cy3, and Cy5 fluorescence were detected using bandpass filter HQ515/30, longpass filter HQ660LP, respectively.

Cell Surface Radioiodination, Immunoprecipitation, and N-Glycosidase F Digestion. Cells (10⁷) were washed with PBS, resuspended in 0.5 ml of PBS containing 0.5 mg/ml of sulfo-succinimidyl-3-(4-hydroxyxenyl) propionate (SHP) (Bolton-Hunter reagent; Pierce Chemical Co.), and incubated on ice for 30 min. The reaction was stopped by diluting the cells with 10 ml of 10 mM 1-lysine (Sigma-Aldrich) in PBS. Cells were centrifuged, resuspended in 150 µl of PBS, and 125I-labeled by the lactoperoxidase method. In brief, 1 mCi Na125I (Amersham Pharmacia Biotech) and 30 µl of 140 IU/ml lactoperoxidase (Sigma-Aldrich) solution were added to the cells, and 10 µl aliquots of 0.06% H₂O₂ solution were then added three times, at 7-min intervals. The reaction was stopped by adding 20 mM KI (Sigma-Aldrich) and 1 mM 1-tyrosine (Sigma-Aldrich) in PBS. Subsequently, the cells were lysed in 1% Brij 96-containing (Sigma-Aldrich) lysis buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM iodoacetamide, 1 mM PMSF, and 1 µg/ml each of leupeptin, pepstatin, and aprotinin) and centrifuged for 14,000 rpm for 30 min at 4°C. The supernatants were precleared three times with normal mouse serum (NMS) IgGs coupled to protein A/G-Sepharose beads (Amersham Pharmacia Biotech) and subjected to immunoprecipitation with the indicated Abs coupled to protein A/G-Sepharose beads. The following Abs were used: the 6D6 anti–human TCR Vα12 mAb (27); the UCHT1 anti–human CD3ε mAb (32); the 448 anti–human TCR-γ, rabbit antiserum, generously provided by Dr. B. Alarcón (Centro de Biología Molecular Severo Ochoa, Madrid, Spain); and the CT-1, anti–human pTα rabbit antiserum, generated in this study. The immunoprecipitates were washed five times with 1% Brij 96–containing lysis buffer and, when indicated, digested overnight with N-glycosidase F (N-Gly; 0.2 U/sample; Roche Diagnostics). For analysis, the immunoprecipitates were separated by SDS-12% PAGE under nonreducing conditions or, alternatively, by two-dimensional gels using SDS-10% polyacrylamide tube gels in the first dimension (nonreduced), which were subsequently resolved by SDS-12% PAGE in the second dimension (reduced).

Immunofluorescence and Confocal Microscopy. 48 h after transfection, COS cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin in PBS containing 1% BSA, and stained either with the PC61 fluorescein-labeled anti–mouse CD25 mAb (BD Pharmingen), or with the HP2.6 anti–human CD4 mAb (provided by Dr. F. Sánchez-Madrid, Hospital de la Princesa, Madrid, Spain), followed by goat anti–mouse fluorescein-coupled F(ab')₂ IgGs (Caltag Laboratories). The coverslips were viewed using a Radiance 2000 confocal microscope (Bio-Rad Laboratories). For confocal analysis, pTα-EGFP SUP-T1 stable transfectants were adhered to Poly L-Lys (Sigma-Aldrich) precoated coverslips (5 x 10⁵ cells/coverslip). Coverslips were then washed in PBS, fixed with 2% paraformaldehyde in PBS for 10 min, permeabilized for 5 min with 0.05% Triton X-100 (Sigma-Aldrich), and blocked with 2% BSA/PBS. Cells were then consecutively stained with the anti–TCR-β F(ab')₂ rabbit antibody (provided by Dr. M. Brenner) in combination with Cy5-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories), and with a rabbit anti-CD4 antibody against the ER resident protein PDI (protein disulfide isomerase; StressGen Biotechnologies) plus Cy3-conjugated goat anti–rabbit IgG (Jackson ImmunoResearch Laboratories). Confo- cal microscopy was performed on a Radiance 2000 (Bio-Rad Laboratories) system coupled to an Axiovert S100TV inverted microscope (ZEISS). Fluorescein and EGFP, Cy3, and Cy5 fluorescence were detected using bandpass filter HQ515/30, longpass filter HQ660LP, respectively.

Metabolic Labeling, Immunoprecipitation, and Endoglycosidase H Treatment. 48 h after transfection, COS cells were rinsed in PBS twice and incubated for 30 min in DMEM without l-cysteine and l-methionine. Then, 500 µCi [35S]methionine/cysteine (Amersham Pharmacia Biotech) were added, and cells were pulsed for 30 min after chase periods, in the presence of DMEM supplemented with 10% FCS. Finally, the cells were washed in PBS, lysed in 1% Triton X-100 (Sigma-Aldrich) lysis buffer, and immunoprecipitated either with the PC61 anti–mouse CD25 mAb (BD Pharmingen), with the CT-1 rabbit antiserum against the human pTα tail, or with the HP2.6 anti–
Results

Low Surface Pre-TCR Expression Is pTα Chain Dependent. As shown by flow cytometry using an anti-pTα antiserum and an anti-CD3 mAb (16), the human pre-TCR is expressed on the surface of primary pre-T cells at very low levels, compared with expression levels of the mature TCR-α/β on SP thymocytes (Fig. 1 A) or peripheral T cells (not shown). Low pre-TCR expression such as that found on primary pre-T cells was detected also on a pre-T cell line, SUP-T1 (Fig. 1 B), which expresses TCR-β and pTα chains but lacks TCR-α (31). It is thus possible that, as proposed for the pre-BCR (1, 17), the pre-TCR is expressed at low surface levels due to regulatory mechanisms that operate selectively at early developmental stages. In that case, one would expect that only limited amounts of a conventional mature TCR-α/β could reach the plasma membrane of pre-T cells. In contrast, we found that the introduction of a conventional TCR α chain (Vα12.1) into SUP-T1 pre-T cells (αwt) resulted in a reproducible increase (∼15-fold) in surface expression levels of CD3, presumably through the formation of heterodimers composed of the transfected TCR-α paired with the endogenous TCR-β and associated with CD3. Accordingly, CD3 was found coexpressed in stoichiometric amounts with the TCR-α/β on stable SUP-T1 αwt transfectants (Fig. 1 B).

It remains possible that one component of the low level pre-TCR expression could be limiting amounts of the endogenous pTα chain, whereas increased TCR-α/β surface density on αwt transfectants could be the result of TCR-α overexpression. To rule out that possibility, a pTα–GFP chimeric construct (16) was introduced into SUP-T1 cells, and surface expression of CD3 was analyzed by flow cytometry on stable pTα–GFP transfectants traced by their GFP expression. As shown in Fig. 1 C, GFP+ cells displayed surface CD3 levels that were indistinguishable from those observed on nontransfected SUP-T1 cells, but still 15-fold lower than CD3 levels on αwt transfectants. Moreover, surface CD3 was coexpressed with the pTα–GFP chimeric protein (Fig. 1 B) and with the endogenous TCR-β chain (16) in stoichiometric amounts, indicating that pTα overexpression does not alleviate endogenous pre-TCR components from the regulatory mechanisms that control their limited expression on the surface of pre-T cells. We thus concluded that pTα amounts are not rate limiting for the assembly and expression of the pre-TCR on the surface of SUP-T1 pre-T cells.

Reciprocal experiments aimed at analyzing surface pre-TCR expression levels on mature T cells were then performed by introduction of pTα or TCR-α into a TCR-α–deficient mutant (JR3.11) derived from the mature T cell line Jurkat (30). As shown in Table I, low pre-TCR and high TCR-α/β surface expression levels, identical to those observed on SUP-T1 pre-T cells, were consistently detected on JR3.11 T cells. These data indicate that differences in surface expression levels of the pre-TCR and the TCR are not intrinsic to the particular cell type in which these receptors are expressed, but may be due to the presence of a pTα chain in the former and of a TCR α chain in the latter.

The Cyto Tail of pTα Is Sufficient to Reduce Surface Expression of a Conventional TCR α Chain to pTα Expression Levels. To uncover the structural properties of the pTα molecule that might account for the low surface expression of the pre-TCR, TCR-α–pTα chimeric chains were generated as illustrated in Fig. 2 A, by replacing the Cyto, TM, and EC Ig-like constant (C) domains of a TCR α chain

Figure 1. Flow cytometry analysis of human thymocytes and SUP-T1 transfectants. (A) Primary human pre-T cells, isolated ex vivo as described in Materials and Methods, were analyzed by flow cytometry for surface expression of pTα and CD3 using a rabbit anti–human pTα Ab plus a fluorescein-conjugated second reagent and a PE-labeled anti-CD3 mAb. Phenotypic analysis of SP thymocytes was performed on electronically gated CD3+ large-sized thymocytes stained for surface expression of a mature CD3-associated TCR-α/β with an anti-TCR-α/β mAb plus a fluorescein-conjugated second reagent and a PE-labeled anti-CD3 mAb. (B) SUP-T1 pre-T lineage cells were mock transfected (SUP-T1) or transfected either with a human TCR-α (Vα12.1) cDNA (αwt) or with a pTα–GFP cDNA (pTα–GFP). Stable transfecants were analyzed by flow cytometry for the coexpression of pTα and CD3 or TCR-α/β and CD3 as described above. (C) Relative levels of surface CD3 expressed on mock-transfected SUP-T1 (shaded histogram) are compared with those expressed on TCR-α (αwt) transfecants (thin line) or pTα–GFP (bold line) transfecants. Background values (dotted line) were obtained with an isotype-matched irrelevant PE-coupled mAb.

human CD4 mAb. The immunoprecipitates were digested overnight with endoglycosidase H (endo-H; Roche) or left undigested, and resolved by SDS-12% PAGE under reducing conditions. Signal intensity was quantitated by densitometry (Bio-imaging BAS 1500; Fujifilm).
TCR-α-deficient pre-T cell (SUP-T1) and mature T cell (JR3.11) lines were stably transfected either with a wild-type TCR-α construct (αwt) or with the TCR-α–pTα αII or αIII chimeric constructs described in Materials and Methods. SUP-T1 and JR3.11 transfectants were analyzed by flow cytometry for the surface expression levels of the conventional TCR-α/β and those of the chimeric TCR by staining with either a mAb against the Vα12.1 domain shared by the TCR-α and TCR-α–pTα chains, or with an anti-CD3 mAb, respectively. Levels of surface expression of endogenous pre-TCR on SUP-T1 pre-T cells were compared to pre-TCR levels on pTα transfectants derived from JR3.11 mature T cells by staining with a rabbit anti-human pTα Ab (reference 16) and flow cytometry. Data are presented as the mean of fluorescence intensity (FL) ± SD of four to six individual transfectants.

|                | SUP-T1 (mean of FL ± SD) | JR3.11 (mean of FL ± SD) |
|----------------|--------------------------|---------------------------|
| pTα            | 1.2 ± 0.5                | 1.5 ± 0.7                 |
| αwt            | 14.1 ± 2.0               | 13.3 ± 2.0                |
| αII            | 1.3 ± 0.4                | 1.4 ± 0.3                 |
| αIII           | 2.2 ± 0.5                | 2.4 ± 0.8                 |

Table I. Relative Levels of Surface pre-TCR, Mature TCR-α/β, and Chimeric TCR Expression on Stable Transfectants Derived from Human Pre-T Cell and Mature T Cell Lines

Figure 2. The Cyto tail of pTα is sufficient to reduce levels of surface expression of a conventional TCR α chain to pTα expression levels. (A) Schematic representation of the three chimeric TCR-α–pTα chains (αII, αIII, and αII), and of the wild-type TCR-α (αwt) and pTα (pTα) chains. (B) Stable transfectants derived from SUP-T1 human pre-T cells were selected in the presence of G-418, and analyzed by flow cytometry for surface expression levels of the αI, αII, or αIII TCR-α–pTα chimeric chains or the wild-type TCR-α chain with an anti-Vα12.1 mAb plus a PE-labeled goat anti-mouse IgG1 (shaded histograms). Background staining (unshaded histograms) was determined with a nonreactive mouse IgG1 mAb plus PE-coupled goat anti-mouse IgG1. Results are representative of at least 10 different stable transfectants. (C) Mean fluorescence values ± SD of surface expression of TCR-α (αwt) and αII and αIII chimeric chains on three distinct stable transfectants of each type. Results are representative of 60–70 distinct transfectants of each type and summarize the data from six experiments.
this effect could be attributed to defective TCR–CD3/ζ interactions, we directly compared the biochemical composition of chimeric TCRs with that of conventional TCR-α/βs, and pre-TCR complexes containing a wild-type pTα chain. To this end, the αwt, αII, and αIII stable transfectants were surface iodinated, treated with 1% Brij 96–containing lysis buffer, and surface TCRs were immunoprecipitated from cell lysates with a mAb specific for their shared Vα12.1 domain. The untransfected SUP-T1 pre-T cell line was simultaneously labeled and the endogenous pre-TCR complex was immunoprecipitated from cell lysates with a rabbit antisera (CT-1) raised against a synthetic peptide contained in the Cyto region of the human pTα molecule. Specificity of the affinity-purified Ab was confirmed previously by immunofluorescence microscopy of COS cells transfected with a c-myc–tagged pTα chain (data not shown). Subsequently, the immunoprecipitates were mock treated or treated with N-Gly, in order to distinguish between CD3ε and CD3δ chains, and resolved by SDS-PAGE under nonreducing conditions. As shown in Fig. 3, a conventional TCR-α/β heterodimer of 90 kD was coimmunoprecipitated with CD3γδε from the surface of αwt transfectants, whereas fewer CD3γδε-associated chimeric TCRs of an apparent MW of 115 kD were immunoprecipitated from both αII and αIII transfectants. N-Gly treatment of the immunoprecipitated proteins resulted in an increase in their electrophoretic mobility by elimination of their glycosidic component, and revealed no qualitative biochemical differences in the composition of the CD3 modules associated with conventional and chimeric TCRs, as all γ, δ, and ε CD3 components were reproducibly precipitated with both heterodimers. Similarly, CD3γ, CD3δ, and CD3ε chains were all associated with a pTα-containing complex of 100 kD immunoprecipitated from the surface of untransfected SUP-T1 pre-T cells, corresponding to the endogenous pre-TCR. However, coprecipitation of ζ chain components either with the pre-TCR or with the chimeric or the conventional TCR-α/βs was not detected under these experimental conditions, likely...
reflecting poor $\zeta$ chain iodination due to its short EC domain (33).

To directly investigate whether the $\zeta$ chain was physically associated with the pre-TCR and the chimeric TCRs as with the conventional TCR-$\alpha$/$\beta$, cell lysates from surface-iodinated SUP-T1 pre-T cells and $\alpha$wt and $\alpha$III transfectants were immunoprecipitated in parallel either with a rabbit anti-human $\zeta$ chain antiserum or with an anti-CD3 $\varepsilon$ mAb, and the immunoprecipitates resolved by two-dimensional nonreducing/reducing SDS-PAGE. As shown in Fig. 4, the anti-CD3 $\varepsilon$ mAb coprecipitated the TCR-$\alpha/\beta$, as well as the $\alpha$III chimeric TCR, and the pre-TCR with CD3$\gamma$/$\delta$, whereas no $\zeta$ chain was detected. However, the $\zeta$ chain was detected as an individual protein migrating out of the diagonal with an apparent MW of 16 kD in anti-$\zeta$ immunoprecipitates. Interestingly, in all samples the anti-$\zeta$ Ab coprecipitated an heterodimeric complex together with CD3$\gamma$/$\delta$, indicating that the $\zeta$ chain is physically associated at the cell surface with the pre-TCR and with the $\alpha$III chimeric TCR, as observed with the mature TCR-$\alpha/\beta$. Furthermore, it is worth noting that the wild-type pre-TCR was precipitated with both the anti-CD3 $\varepsilon$ and anti-$\zeta$ Abs not only from the untransfected SUP-T1 cells, but also from the surface of $\alpha$wt and $\alpha$III SUP-T1 transfectants (Fig. 4), indicating that the conventional TCR $\alpha$ chain (or the $\alpha$III chimeric chain) does not prevent surface expression of the endogenous pre-TCR but, rather, both the pre-TCR and the TCR-$\alpha/\beta$ can be coexpressed at the cell surface.

The Cyto Tail of $p\tau$ Functions As an ER Retention Signal. As biochemical studies provided evidence that low surface expression of the pre-TCR and the chimeric TCRs does not result from their impaired association with CD3/$\zeta$ components (although quantitative differences can not be ruled out), we next investigated whether the p$\tau$ Cyto tail by itself could be directly responsible for controlling surface expression levels of the whole receptor complex. To test this possibility, we first analyzed the effect of the p$\tau$ Cyto domain on the surface expression levels of individual plasma membrane reporter proteins. Murine CD25 (IL-2 receptor $\alpha$ chain) is a particularly useful marker in this type of study, because transfection of the wild-type protein results in high surface expression level, as assessed by flow cytometry with an anti-CD25 mAb (Fig. 5). Interestingly, we found that replacement of the CD25 Cyto domain with that of p$\tau$ results in a $>$50-fold reduction of surface CD25 expression on COS cells transfected with the CD25–p$\tau$ chimera, compared with surface expression levels of wild-type CD25. A comparable reduction of CD25 expression was observed in SUP-T1 pre-T cells transfected with the CD25–p$\tau$ chimera (Fig. 5). In both cell types, the relative levels of CD25 expression were not

![Figure 5](image1.png)  
**Figure 5.** Impaired surface expression of a CD25–p$\tau$ chimeric protein bearing the human $p\tau$ Cyto tail. Relative levels of surface expression of murine CD25 were analyzed by flow cytometry (shaded histograms) with a biotin-coupled anti-CD25 mAb plus PE-coupled streptavidin on SUP-T1 pre-T cells or COS cells transfected with either the wild-type murine CD25 (CD25), a truncated CD25 molecule lacking the cytoplasmic domain (CD25 tailless), or a chimeric protein consisting of the CD25 EC and TM domains and the p$\tau$ Cyto tail (CD25/p$\tau$). Mock-transfected cells were analyzed as controls. Background values (unshaded histograms) were determined by staining with a biotin-coupled isotype-matched irrelevant mAb plus PE-coupled streptavidin.

![Figure 6](image2.png)  
**Figure 6.** ER retention of a CD25–p$\tau$ chimeric protein bearing the Cyto tail of human p$\tau$. (A) COS cells were transfected with either cDNA encoding the wild-type murine CD25 or with a CD25–p$\tau$ chimeric cDNA engineered to encode the CD25 EC and TM domains and the p$\tau$ Cyto tail (CD25–p$\tau$). 48 h after transfection, cells were fixed, permeabilized, stained with a fluorescein-conjugated anti-CD25 mAb, and analyzed by immunofluorescence microscopy. Original magnification: $\times$63. (B) CD25 and CD25–p$\tau$ COS transfectants were pulse-chase labeled with [35S]methionine/cysteine and lysed in Triton X-100–containing lysis buffer. CD25 (top) and CD25–p$\tau$ (bottom) immunoprecipitates obtained with an anti-mouse CD25 (PC.61) mAb or with a rabbit antiserum against the human p$\tau$ tail (CT-1), respectively, were treated with endo-$H$ (+) or left untreated (−) and resolved by 12% SDS-PAGE under reducing conditions. The positions of the mature (m) and immature (im) proteins is indicated on the right.
altered by deletion of the CD25 cyto tail (CD25 tailless), confirming that impaired CD25 surface expression was indeed due to the presence of the pTα Cyto tail.

To assess whether the CD25–pTα chimeric protein was retained intracellularly, COS transfectants were permeabilized and examined for the intracellular distribution of CD25 by immunofluorescence microscopy. As shown in Fig. 6 A, transfection of wild-type CD25 resulted in a predominant localization of the protein in the Golgi apparatus, although CD25 expression was also detected at the plasma membrane. However, a markedly different staining pattern showing strong perinuclear fluorescence and a tubulovesicular peripheral staining was observed in COS cells transfected with the CD25–pTα chimera, which strongly suggested an ER distribution. To confirm that the pTα tail was in fact conferring ER residence to membrane proteins, we next examined susceptibility to endo-H digestion of wild-type and chimeric CD25–pTα molecules, as protein transport out of the ER to the Golgi apparatus is marked by acquisition of endo-H resistance (34). To this end, COS transfectants were labeled for 30 min with [35S]methionine and chased for various periods of time up to 8 h, and Triton X-100-solubilized molecules were then subjected to immunoprecipitation with an anti-CD25 mAb or with an antiserum against the pTα Cyto tail (CT-1). Mock-treated or endo-H–treated proteins were then analyzed for their glycosylation state by SDS-PAGE, as endo-H sensitivity causes a marked change in the electrophoretic mobility of CD25. As shown in Fig. 6 B (top), a significant fraction (~40%, as quantified by densitometric analysis) of wild-type CD25 acquired endo-H resistance after 1 h of biosynthesis, and was completely converted after 4 h. The situation was markedly different for the CD25–pTα chimeras analyzed from either anti-pTα (Fig. 6 B, bottom) or anti-CD25 precipitates (data not shown), as none of them acquire endo-H resistance, even after a 4-h chase. In contrast to wild-type CD25, which was partly degraded after 4 h and was hardly detectable after 8 h, up to 20% of pulsed CD25–pTα was still recovered essentially in an immature form after 8 h (Fig. 6 B). Therefore, the lack of maturation of the chimeric protein does not result from their increased degradation rate in the ER. Rather, we can conclude that a major proportion of the CD25–pTα chimeric proteins primarily reside in the ER and, therefore, cannot be processed in the Golgi apparatus. Taken together, the biosynthetic data in conjunction with the flow cytometry and immunofluorescence microscopy analyses provide evidence that the Cyto tail of the human pTα molecule confers ER residency to plasma membrane proteins.

The physiological relevance of these findings was further confirmed by confocal microscopy studies aimed at analyzing the intracellular distribution of the native pTα protein in pre-T cells displaying poor surface expression of the pre-TCR (Fig. 7). When permeabilized SUP-T1 pre-T cells were stained with the rabbit anti-pTα Ab CT-1 in combination with fluorescein-conjugated goat anti-rabbit Igs, native pTα showed a typical intracellular staining compatible with ER location (data not shown). An identical intracellular pattern was revealed by green fluorescence examination of SUP-T1 pre-T cells transfected with a pTα–GFP chimeric protein (Fig. 7). To provide formal proof that the intracellular compartment containing pTα was in fact the ER, double-color analyses were performed in pTα–GFP transfectants stained with a rabbit Ab recognizing the ER resident PDI protein followed by Cy3-conjugated goat anti-rabbit Igs. As shown in Fig. 7, GFP clearly localized to the same intracellular region as the PDI protein in pTα–GFP transfectants. Moreover, costaining with an anti-TCR-β (βF1) mAb followed by Cy5-conjugated goat anti–mouse Igs revealed that endogenous TCR-β colocal-
alyzed with GFP and PDI. Thus, from these experiments we concluded that intracellular pTα and TCR-β are predominately retained in the ER in SUP-T1 pre-T cells.

**Partial Truncation of the pTα Cyto Tail Releases a CD4–pTα Chimeric Protein from ER Retention and Restores Cell Surface Protein Expression.** Collectively, the above experiments provided evidence that the Cyto domain of pTα is directly responsible for retaining proteins in the ER. Thus, it was reasonable to predict that deletion of the pTα tail (and, therefore, the proposed ER retention determinants) would result in enhanced expression of the pre-TCR complex at the plasma membrane. However, the effect of the pTα tail deletion was the opposite, as it prevented surface pre-TCR expression. Indeed, JR3.11 cells transfected with a tailless pTα chain failed to react with anti–CD3 mAbs (Fig. 8 A), as well as with an anti–TCR-β (VB8) mAb and an anti-pTα Ab (data not shown). This unexpected discrepancy could be explained if the cytoplasmic domain of pTα contains essential structural information for the proper assembly and/or surface expression of a CD3-associated pre-TCR.

The failure to observe surface expression of a pre-TCR with a complete deletion of the pTα tail prompted us to define more precisely the location of the potential ER retention signal in the pTα Cyto domain. To this end, we examined the effect of successive truncations along the pTα tail on the intracellular location and surface expression levels of individual CD4–pTα chimeric proteins which could be readily detected by staining with a mAb against CD4. As shown in Fig. 8 B, immunofluorescence microscopy analyses of COS cells transfected with CD4–pTα chimeric constructs where the Cyto domain of human CD4 was replaced with the full pTα tail (CD4–pTα) revealed a clear tubular-vesicular staining of the ER that was virtually identical to that recorded for the CD25–pTα chimera (Fig. 6 A), and correlated with low surface CD4 expression on both COS (data not shown) and BW cells (Fig. 8 B). No loss of the CD4 ER staining pattern was noted with the removal of the 22 COOH-terminal pTα residues by a truncation at position 243 (CD4–pTαt22), although this truncation resulted in a partial increase of surface CD4 expression on the corresponding BW transfectants (Fig. 8 B). In contrast, when an additional 26 amino acids (aa) were removed, the resulting CD4–pTαt48 truncated chimera was expressed on the surface of BW at levels that were as high as those expressed on BW cells transfected with a CD4-tailless (Fig. 8 B) or a wild-type CD4 (data not shown) construct. As this enhanced expression of surface CD4 correlated with loss of the ER staining pattern and appearance of fluorescence staining at the plasma membrane, we concluded that a region in the pTα Cyto domain between aa 217 and 243 is responsible for the observed ER retention function of the pTα tail. Accordingly, the CD4–pTαt48 chimera as well as the CD4-tailless protein were efficiently transported from the ER to the Golgi apparatus, and displayed similar kinetics, as assessed by pulse-chase metabolic labeling of COS transfectants, anti-CD4 immunoprecipitation, and endo-H treatment. As
particular truncation may release pTα from ER retention, resulting in enhanced surface pre-TCR expression. In contrast, surface expression of a pre-TCR composed of the pTαt22 truncated protein was indistinguishable from expression levels of a wild-type pre-TCR. Collectively, our data suggest that the observed pTα ER retention function contributes to the regulation of surface pre-TCR expression on pre-T cells.

Discussion

Complementary approaches in this study allowed us to conclude that differences between plasma membrane levels of the pre-TCR and the TCR are not intrinsic to the particular cell type in which they are expressed, but depend on the presence of a pTα chain in the former and of a TCR-α chain in the latter. We have first shown that introduction of a conventional TCR-α chain into pre-T cells increases surface receptor expression to levels similar to those of TCR-α/β on transfected mature T cells. Interestingly, although it has been reported that expression of TCR-α precludes the formation of a TCR-β–pTα dimer in a murine pre-T cell line (35), TCR-α transfection does not impair endogenous pre-TCR surface expression on human pre-T cells, indicating that TCR-β chains are in large excess and that TCR-α and pTα are not competing each other for pairing with TCR-β. Conversely, transfection of a TCR-α-deficient mature T cell line with a pTα chain results in low surface expression of the pre-TCR, relative to TCR-α/β surface levels induced upon TCR-α transfection. Because low levels of pre-TCR expression is a common feature of both cell types, we concluded that this is an intrinsic property of the pre-TCR complex, and therefore, the pTα chain itself might play a direct role in controlling pre-TCR expression levels.

In an effort to identify the structural domain/s of pTα responsible for such a role, we took advantage of the similarities and differences between the EC, TM, and Cyto domains of pTα and TCR-α and produced TCR-α–pTα chimeric proteins that were analyzed for their ability to bring a CD3–associated chimeric TCR complex to the cell surface. We initially focused on the EC pTα region, which lacks a covalently associated Ig-like V domain (25), and asked the question of whether replacement of the pTα EC domain with the EC region of TCR-α could provide a symmetrical shape to the TCR-β–pTα heterodimer that could result in an increased expression at the cell surface. Unexpectedly, however, such chimeric TCR-α–pTα chains (αII) did not affect the levels of expression of the pre-TCR, indicating that low surface pre-TCR expression is not dependent on the pTα EC domain. From these data one could infer that the lack in pTα of a partner domain capable of pairing with the hydrophobic surface of the Vβ domain (36) does not impair expression of the complex at the cell surface. Alternatively, the pre-TCR, by analogy with the pre-BCR, may contain an additional not yet identified VpreT component that fulfills that function, and that might thus be rate limiting for proper assembly and

![Figure 9](image-url)
transport of the TCR-β–pTα heterodimer to the cell sur-
face (2, 6). Expression of such a putative VpreT is expected
to be developmentally regulated, such that its absence
would account for the impaired surface expression of the pre-TCR described in murine mature T cells (6). However, hu-
manned mature T cells proved to be as efficient as pre-T cells in expressing a functional TCR-β–pTα heterodimer on
their surface (this study and unpublished results), thus sup-
porting the alternative hypothesis that the pTα chain is suf-
ficient to promote surface expression of the pre-TCR complex in the absence of an additional component.

The finding that the chimeric chain in which the pTα EC domain was replaced with the EC TCR-α domain be-
vales as a wild-type pTα chain in terms of surface receptor expression was not obvious, because the TCR-α C domain
displays little homology with the equivalent pTα domain (9, 25). However, as the TCR-α C domain residues in-
volved in polar interactions with the TCR-β C domain are all conserved in the pTα C domain, it has been proposed
that association between pTα and TCR-β C domains may occur in a way similar to that observed in the TCR CaαCB module (3, 37). These data are against an essential function of TCR-β–pTα assembly in controlling surface expression levels of the pre-TCR, and suggest that the CD3ζ/ζ sub-
units associated with the TCR-β–pTα heterodimer probably account for such a regulatory function. Of them, the ζ chain would merely be a dispensable amplification module for the pre-TCR, which increases its assembly rate and sta-
bility (19–22, 24), and CD3ζ seems dispensable for pre-
TCR assembly and function (8, 13, 38). In contrast, the CD3γζ pair, which may associate with the pre-TCR EC
domain module in a manner similar to that suggested for its association with the TCR-α/β (39), has proved mandatory
for proper assembly of a functional pre-TCR (8, 40–42).

Therefore, it is not surprising that the wild-type pTα chain and the chimeric chains containing both the EC and TM
domains, or exclusively the EC domain of TCR-α, are equally capable of mediating association with CD3γζ, CD3ζ, and CD3ζε subunits. What is more surprising is that all the wild-type pre-TCR and the chimeric TCR complex es-
are equally found physically associated with the ζ chain, given that the weak association of the ζ chain with the pre-TCR has recently been shown to map to the con-
necting peptide (CP) of pTα lying between its TM and Ig-
like EC domains (43). Therefore, one would expect that
the presence of the TCR-α CP domain in both chimeras
would confer an increased stability to their corresponding receptors that might result in higher levels of surface
expression. In contrast, their expression levels were as low as those of the wild-type pre-TCR. Taken together, our re-

isults allowed us to conclude that qualitative differences in
the contribution of the CD3ζ/ζ components to pre-TCR
or TCR assembly do not influence surface receptor expres-
sion levels.

A striking finding was that replacing the Cyto domain of TCR-α with the equivalent pTα domain is sufficient for
inducing low surface expression of the chimeric TCR. It is
thus obvious that the pTα Cyto tail is directly responsible
for low surface pre-TCR expression. A strong argument in
favor of the attractive possibility that the pTα Cyto tail
functions as an ER retention signal was the observation
that individual plasma membrane proteins are mostly re-
tained in the ER, and fail to reach the Golgi compartment
when appended to the pTα tail. Accordingly, pTα chains
are primarily found in internal structures which colocalize
with the ER. The demonstration that truncation of the ter-
minus 48 aa of pTα (and particularly deletion of the region
between residues 217 and 243) releases CD4–pTα chi-
meric proteins from ER retention lends further support to
that notion, and suggests that pTα ER retention deter-
minants localize primarily in the deleted region. However,
the pTα tail sequence from position 217 to 243 does not
harbor the consensus dilysine cytoplasmic motif for ER re-
tention of TM proteins (34) or the reported CD3ζ ER re-
tention sequence (44), suggesting the existence of alterna-
tive ER retention signals. An extensive series of point
mutations will have to be introduced into that particular
pTα sequence to unravel the precise requirements for its retention in the ER.

As proposed for the mature TCR-α/β (45), ER retention
of individual subunits or partial complexes might be an
essential mechanism for regulating assembly and levels of
expression of the pre-TCR. However, additional structural
information may be contained in the pTα Cyto tail that is
essential for surface expression of the pre-TCR, as pTα
tailless molecules fail to bring a CD3-associated pre-TCR
complex to the cell surface. Therefore, efficient surface ex-
pression of the pre-TCR complex may be the result of a
balance between complex mechanisms controlling reten-
tion and assembly of its individual components. This may
explain the finding that release of individual chimeras bear-
ing a truncated pTα tail from ER retention correlates with
recovery of wild-type protein expression levels, whereas
pTα molecules with identical truncations were unable to
recover TCR-α/β surface levels, although they were more
efficient than wild-type pTα in bringing the pre-TCR to
the cell surface. Interestingly, an ER retention mechanism
has also been proposed to account for the low plasma
membrane expression of the pre-BCR (17), although in
that case ER retention seems to be inherent to pre-B cells.

Despite the striking functional similarities between the pre-
TCR and the pre-BCR, the particular structural features of
their individual components (i.e., pTα and λ5) may explain
such a discrepancy.

Finally, although one would expect that a retention
mechanism similar to that proposed here for the human
pre-TCR would be functional also in mice, the poor con-
servation of the Cyto tail in both species (6, 25) would
make this possibility unlikely. However, conserved resi-
dues in the cytoplasmic domain have been identified (25).
In addition, two independent findings strengthen our re-
view: first, DN thymocytes from pTα–/– mice made
transgenic for a pTα tailless chain display increased
TCR-β surface levels, compared with DN thymocytes
from wild-type or pTα–/– nontransgenic mice (46), and
second, low CD3 expression is common to DP thy-
mocytes from recombination activating gene (Rag)−/− mice expressing a transgenic functional pre-TCR consisting of truncated TCR-β and pTα forms that keep their TM and Cyto regions (14). In conclusion, our data provide evidence that the pTα Cyto tail displays a novel ER retention function which may participate in the regulation of surface pre-TCR expression levels on pre-T cells.

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References

1. Borst, J., H. Jacobs, and G. Brouns. 1996. Composition and function of T-cell receptor and B-cell receptor complexes on precursor lymphocytes. Curr. Opin. Immunol. 8:181–190.

2. von Boehmer, H., and H.J. Fehling. 1997. Structure and function of the pre-T cell receptor. Annu. Rev. Immunol. 15: 433–452.

3. Malissen, B., L. Ardouin, S.-Y. Lin, A. Gillet, and M. Malissen. 1999. Function of the CD3 subunits of the pre-TCR and TCR complexes during T cell development. Adv. Immunol. 72:103–148.

4. Groettrup, M., K. Ungeweiss, O. Azogi, R. Palacios, M.J. Owen, A.C. Hayday, and H. von Boehmer. 1993. A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor β chain and a 33 kd glycoprotein. Cell. 75:283–294.

5. Dudley, E.C., H.T. Petrie, L.M. Shah, M.J. Owen, and A.C. Hayday. 1994. T cell receptor β chain gene rearrangement and selection during thymocyte development in adult mice. Immunity. 1:83–93.

6. Saint-Ruf, C., K. Ungeweiss, M. Groettrup, L. Bruno, H.J. Fehling, and H. von Boehmer. 1994. Expression and analysis of a cloned pre-T cell receptor gene. Science. 266:1208–1212.

7. van Oers, N.S.C., H. von Boehmer, and A. Weiss. 1995. The pre-T cell receptor (TCR) complex is functionally coupled to the TCR-β subunit. J. Exp. Med. 182:1585–1995.

8. Berger, M.A., V. Davé, M.R. Rhodes, G.C. Bosma, M.J. Bosma, D.J. Kappes and D.L. Wiest. 1997. Subunit composition of pre-T cell receptor complexes expressed by primary thymocytes: CD3δ is physically associated but not functionally required. J. Exp. Med. 186:1461–1467.

9. Fehling, H.J., A. Krotkova, C. Saint-Ruf, and H. von Boehmer. 1995. Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. Nature. 375:795–798.

10. Hoffman, E.S., L. Passoni, T. Crompton, T.M.J. Leu, D.G. Schatz, A. Koff, M.J. Owen, and A.C. Hayday. 1996. Productive T-cell receptor beta-chain gene rearrangement and selection during thymocyte development in vivo. Genes Dev. 10:948–962.

11. Malissen, B., and M. Malissen. 1996. Functions of TCR and pre-TCR subunits: lessons from gene ablation. Curr. Opin. Immunol. 8:383–393.

12. Leveilt, C.N., B. Wang, A. Ehrfeld, C. Terhost, and K. Eichmann. 1995. Regulation of T cell receptor (TCR)-β locus allelic exclusion and initiation of TCR-α locus rearrangement in immature thymocytes by signaling through the CD3 complex. Eur. J. Immunol. 25:1257–1261.

13. Jacobs, H., D. Vandeputte, L. Tolkamp, E. de Vries, J. Borst, and A. Berns. 1994. CD3 components at the surface of pro-T cells can mediate pre-T cell development in vivo. Eur. J. Immunol. 24:934–939.

14. Irving, B.A., F.W. Alt, and N. Killeen. 1998. Thymocyte development in the absence of pre-TCR cell receptor extracellular immunoglobulin domains. Science. 280:905–908.

15. O’Shea, C.C., A.P. Thornell, I.R. Rosewell, B. Hayes, and M.J. Owen. 1997. Exit of the pre-TCR from the ER/cis-golgi is necessary for signaling differentiation, proliferation, and allelic exclusion in immature thymocytes. Immunity. 7:591–599.

16. Tringueiros, C., A.R. Ramiro, Y.R. Carrasco, V.G. de Yébenes, J.P. Albar, and M.L. Toribio. 1998. Identification of a late stage of small noncycling pTα− pre-T cells as immediate precursors of T cell receptor α/β+ thymocytes. J. Exp. Med. 188:1401–1412.

17. Brouns, G.S., E. de Vries, J.J. Neeftjes, and J. Borst. 1996. Assembled pre-B cell receptor complexes are retained in the endoplasmic reticulum by a mechanism that is not selective for the pseudo-light chain. J. Biol. Chem. 271:19272–19278.

18. Jacobs, H., F. Osendorp, E. de Vries, K. Ungewiss, K., H. von Boehmer, J. Borst, and A. Berns. 1996. Oncogenic potential of a pre-T cell receptor lacking the TCRβ variable domain. Oncogene. 12:2089–2099.

19. Malissen, M., A. Gillet, B. Rocha, J. Trucy, E. Vivier, C. Boyer, C. Köntgen, N.K. Brun, G. Mazza, E. Spanopoulou, D. Guy-Grand, and B. Malissen. 1993. T cell development in mice lacking the CD3ζ/η gene. EMBO (Eur. Mol. Biol. Organ.) J. 12:4347–4355.

20. Love, P.E., E.W. Shores, M.D. Johnson, M.L. Tremblay, E.J. Lee, A. Grinberg, S.P. Huang, A. Singer, and H. Westphal. 1993. T cell development in mice that lack the zeta chain of the T cell antigen receptor complex. Science. 261: 918–921.

21. Ohno, H., T. Aoe, S. Taki, D. Kitamura, Y. Ishida, K. Rajewski, and T. Saito. 1993. Developmental and functional impairment of T cells in mice lacking CD3ζ chains. EMBO (Eur. Mol. Biol. Organ.) J. 12:4357–4366.

22. Shores, E.W., K. Huang, T. Tran, E. Lee, A. Grinberg, and P.E. Love. 1994. Role of TCR-ζ chain in T cell development and selection. Science. 266:1047–1050.

23. Bäckström, B.T., E. Mila, A. Peter, B. Jauregulber, C.T. Baldari, and E. Palmer. 1996. A motif within the T cell receptor ζ chain constant region connecting peptide domain controls antigen responsiveness. Immunity. 5:437–447.

24. Ardouin, L., J. Ismaili, B. Malissen, and M. Malissen. 1998. The CD3-γδε and CD3-ζη modules are each essential for allelic exclusion at the T cell receptor β locus but are both dispensable for the initiation of V to (DJ) recombination at
the T cell receptor-β, -γ, and -δ loci. J. Exp. Med. 187:105–116.

25. Ramiro, A.R., C. Trigueros, C. Márquez, J.L. San Millán, and M.L. Toribio. 1996. Regulation of pre-T cell receptor (pTα-TCRβ) gene expression during human thymic development. J. Exp. Med. 184:519–530.

26. Borst, J., J.J.M. van Dongen, E. de Vries, W.M. Comans-Bitter, M.J.D. van Tol, J.M. Vossen, and R. Kurrle. 1990. BMA031, a monoclonal antibody suited to identify the T-cell receptor αβ/CD3 complex on viable human T lymphocytes in normal and disease states. Hum. Immunol. 29:175–188.

27. DerSimonian, H., H. Band, and M.B. Brenner. 1991. Increased frequency of T cell receptor Vα12.1 expression on CD8* T cells: evidence that Vα participates in shaping the peripheral T cell repertoire. J. Exp. Med. 174:639–648.

28. Maddon, P.J., D.R. Littman, M. Godfrey, D.E. Maddon, L. Chess, and R. Axel. 1985. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. Cell. 42:93–104.

29. Heemskerk, M.H.M., B. Bloom, G. Nolan, A.P.A. Stegmann, A.Q. Bakker, K. Weijer, P.C.M. Res, and H. Spits. 1997. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. J. Exp. Med. 186:1597–1602.

30. Arnaud, J., A. Huchenq, M.-C. Vernhes, S. Caspar-Bauguil, F. Lenfant, J. Sancho, C. Terhost, and B. Rubin. 1996. The interchain disulfide bond between TCRαβ heterodimers on human T cells is not required for TCR-CD3 membrane expression and signal transduction. Int. Immunol. 9:615–626.

31. Reynolds, T.C., S.D. Smith, and J. Sklar. 1987. Analysis of DNA surrounding the breakpoints of chromosomal translocations involving the beta T cell receptor gene in human lymphoblastic neoplasms. Cell. 50:107–117.

32. Wallace, D.L., E.A. Machutrye, D.C. Linch, and P.C.L. Beverley. 1987. Analysis of the activation signals induced by CD3 antibodies and their role in T cell activation. In Leucocyte Typing III. Oxford University Press, Oxford, UK. 167–169.

33. Weissmann, A.M., M. Baniyah, D. Hou, L.E. Samelson, W.H. Burgess, and R.D. Klausner. 1988. Molecular cloning of the zeta chain of the T cell antigen receptor. Science. 239:1018–1021.

34. Jackson, M.R., T. Nilsson, and P.A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO (Eur. Mol. Biol. Organ.) J. 9:3153–3162.

35. Trop, S., M. Rhodes, D.L. Wiest, P. Hugo, and J.C. Zúñiga-Pflücker. 2000. Competitive displacement of pTα by TCRα during TCR assembly prevents surface coexpression of pre-TCR and αβ TCR. J. Immunol. 165:5566–5572.

36. Bentley, G.A., G. Boulot, K. Karjalainen, and R.A. Mariuzza. 1995. Crystal structure of the β chain of a T cell antigen receptor. Science. 267:1984–1987.

37. Wang, J.-H., K. Lim, A. Smolyar, M.-K. Teng, J.-H. Liu, A.G.D. Tse, J. Liu, R.E. Hussey, Y. Chishti, C.T. Thompson, et al. 1998. Atomic structure of an αβ T cell receptor (TCR) heterodimer in complex with an anti-TCR. Fab fragment derived from a mitogenic antibody. EMBO (Eur. Mol. Biol. Organ.) J. 17:10–26.

38. Dave, P.D., Z. Cao, C. Browne, B. Alarcón, G. Fernández-Miguel, J. Lafaille, A. de la Hera, S. Tonegawa, and D.J. Kappes. 1997. CD3 delta deficiency arrests development of the alpha beta but not the gamma delta T cell lineage. EMBO (Eur. Mol. Biol. Organ.) J. 16:1360–1370.

39. Manolios, N., O. Kemp, and Z.G. Li. 1994. The T cell antigen receptor α and β chains interact via distinct regions with CD3 chains. J. Immunol. 24:84–92.

40. Malissen, M., A. Gillet, L. Ardouin, G. Bouvier, J. Trucy, P. Ferrier, E. Vivier, and B. Malissen. 1995. Altered T cell development in mice with a targeted mutation of the CD3-ε gene. EMBO (Eur. Mol. Biol. Organ.) J. 14:4641–4653.

41. Haks, M.C., P. Krippenfört, J. Borst, and A. Kruijssen. 1998. The CD3γ chain is essential for development of both the TCRαβ and TCRγδ lineages. EMBO (Eur. Mol. Biol. Organ.) J. 17:1871–1882.

42. Defarnette, J.B., C.L. Sommers, K. Huang, K.J. Woodside, R. Emmons, K. Katz, E.W. Shores, and P.E. Love. 1998. Specific requirement for CD3epsilon in T cell development. Proc. Natl. Acad. Sci. USA. 95:14909–14914.

43. Trop, S., A.M. Steff, F. Denis, D.L. Wiest, and P. Hugo. 1999. The connecting peptide domain of pTα dictates weak association of the pre-T cell receptor with the TCR ζ subunit. Eur. J. Immunol. 29:2187–2196.

44. Mallabiaharreña, A., M. Fresno, and B. Alarcón. 1992. An endoplasmic reticulum retention signal in the CD3ε chain of the T-cell receptor. Annu. Rev. Cell Biol. 6:403–431.

45. Fehling, H.J., B.M. Iritani, A. Krotkova, K.N. Forbush, C. Laplace, R.M. Perlmutter, and H. von Boehmer. 1997. Restoration of thymopoiesis in pTα−/− mice by anti-CD3ε antibody treatment or with transgenes encoding activated lck or telless pTα. Immunity. 6:703–714. 1057  Carrasco et al.