

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

By Yolanda R. Carrasco, Almudena R. Ramiro, César Trigueros, Virginia G. de Yébenes, Marina García-Peydró, and María L. Toribio

From the Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

Abstract

The pre-T cell receptor (TCR), which consists of a TCR- β chain paired with pre-TCR- α (pT α) 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 -pT α proteins into pre-T and mature T cell lines, we show here that the low surface expression of the human pre-TCR is pT α chain dependent. Particularly, the cytoplasmic domain of pT α 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 pT α 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 pT α is also observed to be predominantly ER localized. Finally, sequential truncations along the pT α cytoplasmic domain revealed that removal of the COOH-terminal 48 residues is sufficient to release a CD4-pT α chimera from ER retention, and to restore native CD4 surface expression levels. As such a truncation in pT α also correlates with enhanced pre-TCR expression, the observed pT α ER retention function may contribute to the regulation of surface pre-TCR expression on pre-T cells.

Key words: human pre-T cell receptor • pT α 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 (pT α)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 pT α by TCR- α , 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

C. Trigueros' present address is Imperial Cancer Research Fund, London WC2A 3PX, UK.

Address correspondence to M.L. Toribio, Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain. Phone: 34-91-397-8076; Fax: 34-91-397-8087; E-mail: mtoribio@cbm.uam.es

¹Abbreviations used in this paper: aa, amino acid(s); BCR, B cell receptor; Cyto, cytoplasmic; DN, double negative; DP, double positive; EC, extracellular; EGFP, enhanced green fluorescent protein; endo-H, endoglycosidase H; ER, endoplasmic reticulum; PDI, protein disulfide isomerase; pT α , pre-TCR- α ; SP, single positive; TM, transmembrane.

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 is expressed only transiently during thymocyte development, and at extremely low levels, ~ 50 – 100 -fold lower than those of the TCR- α/β on mature T cells (1, 13, 16). The inefficient expression of pre-TCR complexes on pre-T cells is reminiscent of the inefficient expression of the pre-B cell receptor (BCR) on pre-B cells (1, 17), and might be related to the particular signaling function of pre-TCR/BCR complexes during lymphoid development. The question is, therefore, whether such differences in surface expression levels are determined by specific structural properties of the receptors or are intrinsic to the particular developmental stage at which the receptors are expressed.

Although the precise biochemical composition of 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 pT α 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 -pT α molecules comprising distinct domains of TCR- α and pT α 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 pT α chain. Particularly, our results show that the cytoplasmic (Cyto) domain of pT α is sufficient to promote retention in the ER and to reduce surface expression levels of individual transmembrane (TM) proteins. The possibility that the pT α 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/CD3^{low} pre-T cells were isolated from the large-sized cell fraction by immunomagnetic sorting (Dyna) 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. Kurrle (Behringwerke AG, Marburg, Germany), and the 6D6 mAb, against the human V $\alpha_{12.1}$ TCR- α domain (27), was the gift of Dr. M. Brenner (Brigham and Women's Hospital, Boston, MA). Surface expression of the human pT α chain was determined by staining with a rabbit antiserum (ED-1) raised against a synthetic peptide present in the pT α 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 pT α 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 -pT α chimeric constructs (see Fig. 2 A) comprising the V $\alpha_{12.1}$ and J α domains of the AV12S1 TCR α chain fused to pT α cDNA lacking the leader sequence (α I), or encoding a AV12S1 TCR α chain in which either both the TM and Cyto domains (α II) or exclusively the Cyto domain (α III) of TCR- α have been replaced with homologous domains from pT α , were generated by PCR using TCR- α and pT α cDNAs as templates, and specific oligonucleotides. The sense 5'-ATG GAT CCT CTA GAT 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 TGG TGC CTG TTC CTG TTC-3' (with a BamHI site), 5'-CGG AAT TCG GTT TTG AAA GTT TAG GTT CG-3' (with an EcoRI site), or 5'-CGG AAT TCC CAG CGT CAT GAG CAG ATT AAA-3' (with an EcoRI site), which correspond to the COOH-terminal region of either the J α , 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 pT α lacking the leader sequence, an EcoRI/XhoI PCR fragment encoding both the TM and Cyto pT α domains, or an EcoRI/XhoI PCR fragment encoding the Cyto tail of pT α . Such pT α PCR products were amplified from pT α cDNA with the antisense 5'-CCG CTA ACG AGT CAG GCA GCA GCT CCA GCC TGC AG-3' primer, corresponding to the COOH-terminal region of the pT α 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 GCG-3', with an EcoRI site, or 5'-CGG AAT TCT TAC CTG CAG CTG 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-pT α and CD4-pT α chimeric constructs, comprising the complete EC and TM domains of CD25 or CD4 fused to the pT α Cyto tail, were generated by PCR amplification. CD25 PCR products obtained with the sense 5'-ATG GAT CCA AGA TGG AGC CAC GTC TGC TG-3' primer and the antisense 5'-GCG 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'-GCG AAT TCC GAA GAA 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 pT α Cyto tail described above. Truncated CD4-pT α chimeras lacking either the COOH-terminal 22 (CD4-pT α 22) or 48 (CD4-pT α 48) residues of the pT α Cyto tail were generated essentially as described for CD4-pT α chimeric constructs, except that pT α PCR was performed with the sense primer corresponding to the NH₂-terminal region of the pT α Cyto domain described above, in combination with the antisense 5'-AGA GAG AGA GAG GAT ATC TCA AGC CCT GAG GCG AGA TCT TG-3' or 5'-AGA GAG AGA GAG GAT ATC TCA TAC TGG GCT CCC GGG CTT C-3' primers, for CD4-pT α 22 and CD4-pT α 48, 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 pT α region to generate truncated pT α proteins with identical deletions in the Cyto tail (pT α 22 and pT α 48). EcoRI/EcoRV-digested products were independently ligated into pcDNA3. Both cDNAs as well as full-length pT α 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 pT α cDNA was generated by amplification with the sense primer described previously (16) in combination with the 5'-GGG GGA TCC TAC AGG AGC AGG TCA AAC AG-3' antisense primer, BamHI digested, and ligated into the pCIGFP bicistronic plasmid. Each construct was sequenced directly in pcDNA3. The pT α -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 (31), 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 (Biowhittaker) supplemented with 5% FCS (GIBCO BRL). Transfections were carried out by electroporation as described (16).

Generation of Polyclonal Anti-Human pT α Abs. A rabbit antiserum (CT-1) was generated against a synthetic peptide corresponding to the human pT α sequence 200–212 present in the Cyto domain (25), essentially as described previously for the ED-1 antiserum (16). The specificity of the CT-1 antiserum was assayed by immunofluorescence microscopy of COS cells transfected with a pT α cDNA tagged with a c-myc epitope recognized by the specific 9E10 mAb (unpublished results).

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-hydroxyphenyl) propionate (SHPP) (Boltont-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 L-lysine (Sigma-Aldrich) in PBS. Cells were centrifuged, resuspended in 150 μ l of PBS, and ¹²⁵I-labeled by the lactoperoxidase method. In brief, 1 mCi Na¹²⁵I (Amersham Pharmacia Biotech) and 30 μ l of 140 IU/ml lactoperoxi-

dase (Sigma-Aldrich) solution were added to the cells, and 10 μ l 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 L-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 μ g/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) Igs 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 V α _{12.1} mAb (27); the UCHT1 anti-human CD3 ϵ mAb (32); the 448 anti-human TCR- ζ rabbit antiserum, generously provided by Dr. B. Alarcón (Centro de Biología Molecular Severo Ochoa, Madrid, Spain); and the CT-1, anti-human pT α 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 Igs (Caltag Laboratories). The coverslips were viewed using a Radiance 2000 confocal microscope (Bio-Rad Laboratories).

For confocal analysis, pT α -GFP SUP-T1 stable transfectants were adhered to Poly L-Lys (Sigma-Aldrich) precoated coverslips (5 \times 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 β F1 mAb (provided by Dr. M. Brenner) in combination with Cy5-conjugated goat anti-mouse Igs (Jackson ImmunoResearch Laboratories), and with a rabbit antiserum against the ER resident protein PDI (protein disulfide isomerase; StressGen Biotechnologies) plus Cy3-conjugated goat anti-rabbit Igs (Jackson ImmunoResearch Laboratories). Confocal 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 HQ600/50, and 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 μ Ci [³⁵S]methionine/cysteine (Amersham Pharmacia Biotech) were added, and cells were pulsed for 30 min followed by 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 pT α tail, or with the HP2.6 anti-

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 (Bioimaging BAS 1500; Fujifilm).

Results

Low Surface Pre-TCR Expression Is pT α Chain Dependent. As shown by flow cytometry using an anti-pT α 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

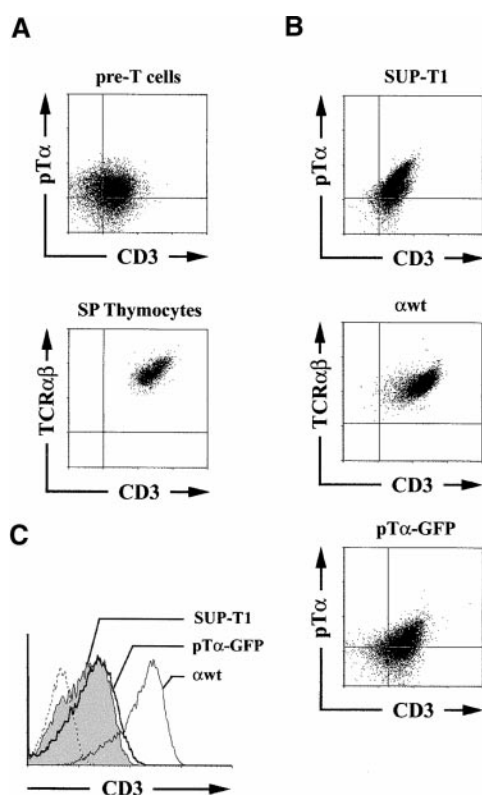


Figure 1. Flow cytometry analysis of human thymocytes and SUP-T1 transfectants. (A) Primary human pre-T cells, isolated ex vivo as described in Materials and Methods, were analyzed by flow cytometry for surface expression of pT α and CD3 using a rabbit anti-human pT α Ab plus a fluorescein-conjugated second reagent and a PE-labeled anti-CD3 mAb. Phenotypic analysis of SP thymocytes was performed on electronically gated CD3^{bright} 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-labeled 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 ($V\alpha_{12.1}$) cDNA (α wt) or with a pT α -GFP cDNA (pT α -GFP). Stable transfectants were analyzed by flow cytometry for the coexpression of pT α 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 (α wt) transfectants (thin line) or pT α -GFP (bold line) transfectants. Background values (dotted line) were obtained with an isotype-matched irrelevant PE-coupled mAb.

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 pT α 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 ($V\alpha_{12.1}$) into SUP-T1 pre-T cells (α wt) resulted in a reproducible increase (\sim 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 α wt transfectants (Fig. 1 B).

It remains possible that one component of the low level pre-TCR expression could be limiting amounts of the endogenous pT α chain, whereas increased TCR- α/β surface density on α wt transfectants could be the result of TCR- α overexpression. To rule out that possibility, a pT α -GFP chimeric construct (16) was introduced into SUP-T1 cells, and surface expression of CD3 was analyzed by flow cytometry on stable pT α -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 α wt transfectants. Moreover, surface CD3 was coexpressed with the pT α -GFP chimeric protein (Fig. 1 B) and with the endogenous TCR- β chain (16) in stoichiometric amounts, indicating that pT α 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 pT α 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 pT α 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 pT α chain in the former and of a TCR α chain in the latter.

The Cyto Tail of pT α Is Sufficient to Reduce Surface Expression of a Conventional TCR α Chain to pT α Expression Levels. To uncover the structural properties of the pT α molecule that might account for the low surface expression of the pre-TCR, TCR- α -pT α 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

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

	SUP-T1 (mean of FL \pm SD)	JR3.11 (mean of FL \pm SD)
pT α	1.2 \pm 0.5	1.5 \pm 0.7
α wt	14.1 \pm 2.0	13.3 \pm 2.0
α II	1.3 \pm 0.4	1.4 \pm 0.3
α III	2.2 \pm 0.5	2.4 \pm 0.8

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 (α wt) or with the TCR- α -pT α α II or α III 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 V α 12.1 domain shared by the TCR- α and TCR- α -pT α 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 pT α transfectants derived from JR3.11 mature T cells by staining with a rabbit anti-human pT α Ab (reference 16) and flow cytometry. Data are presented as the mean of fluorescence intensity (FL) \pm SD of four to six individual transfectants.

bearing a V α _{12.1} domain with homologous domains from the pT α chain. The chimeric constructs were introduced into SUP-T1 pre-T cells, and the resulting stable transfectants were assayed by flow cytometry for surface expression of the chimeric TCRs using a mAb against their shared V α _{12.1} domain. Cells stably transfected with the wild-type TCR α chain (α wt) were analyzed in parallel to control surface expression levels of conventional TCR- α/β s. After screening of up to 70 stable transfectants of each type, no surface expression of the α I chimera consisting of a pT α chain lacking the peptide leader fused to the V α _{12.1} and J α domains of TCR- α was found (Fig. 2 B). However, the α I chimeric protein could be detected intracellularly in COS transfectants (data not shown), thus confirming that the TCR- α modification had not adversely affected protein expression. Therefore, it remains possible that the α I chimera does not associate properly with TCR- β , preventing its surface expression and/or detection. Surprisingly, TCR- α -pT α chimeras in which both the TM and Cyto domains (α II) or exclusively the Cyto domain (α III) of TCR- α were replaced with the equivalent pT α domains were found expressed at the cell surface, although at levels that were reproducibly 10–15-fold lower than those of α wt (Fig. 2 C), but comparable to those of endogenous pT α or transfected pT α -GFP chimeric chains (Fig. 1 C). Importantly, when the α wt and the α II and α III chimeric chains were introduced into the TCR- α -deficient JR3.11 mature T cell line, their relative surface expression levels were identical to those expressed on SUP-T1 pre-T cells (Table I). Taken together, these data provide evidence that replacing the Cyto domain of TCR- α with the pT α Cyto tail is sufficient to reduce TCR α chain surface expression (and,

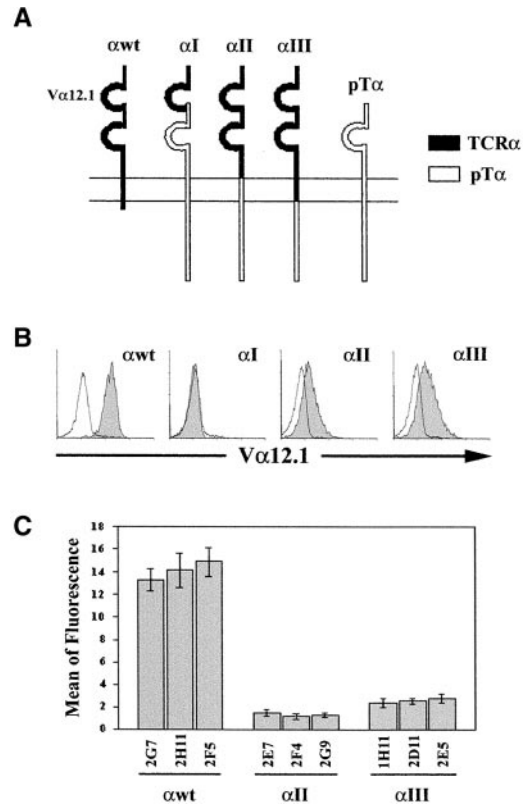


Figure 2. The Cyto tail of pT α is sufficient to reduce levels of surface expression of a conventional TCR α chain to pT α expression levels. (A) Schematic representation of the three chimeric TCR- α -pT α chains (α I, α II, and α III), and of the wild-type TCR- α (α wt) and pT α (pT α) chains. (B) Stable 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 α I, α II, or α III TCR- α -pT α chimeric chains or the wild-type TCR α chain with an anti-V α _{12.1} 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 \pm SD of surface expression of TCR- α (α wt) and α II and α III 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.

hence, expression of the chimeric TCR, see below) to conventional pT α chain expression levels, whereas the TM domains of the TCR- α and pT α chains were equivalent for surface expression of the TCR- α -pT α chimera.

CD3/ ζ Components Are Physically Associated in the Wild-type Pre-TCR as well as in the TCR- α -pT α Chimeric TCRs. Three-color flow cytometry showed that the wild-type TCR α chain and the α II and α III (α I was not studied further) chimeric proteins were expressed at the cell surface in stoichiometric amounts with TCR- β (V β 1.1) and CD3 (data not shown), indicating that surface expression of α wt, α II, and α III chains must be achieved by pairing them with the endogenous TCR- β chain and CD3/ ζ components. Therefore, the cyto tail of pT α appears sufficient to reduce surface expression of the chimeric TCR complex to pre-TCR expression levels. To assess whether

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/β s, and pre-TCR complexes containing a wild-type pT α chain. To this end, the α wt, α II, and α III 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 V $\alpha_{12.1}$ 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 pT α molecule. Specificity of the affinity-purified Ab was confirmed previously by immunofluorescence microscopy of COS cells transfected with a c-myc-tagged pT α chain (data not shown). Subsequently, the immunoprecipitates were mock treated or treated with N-Gly, in order to distinguish between CD3 ϵ and CD3 δ 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 CD3 $\gamma\delta\epsilon$ from the surface

of α wt transfectants, whereas fewer CD3 $\gamma\delta\epsilon$ -associated chimeric TCRs of an apparent MW of 115 kD were immunoprecipitated from both α II and α III 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, CD3 γ , CD3 δ , and CD3 ϵ chains were all associated with a pT α -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/β s was not detected under these experimental conditions, likely

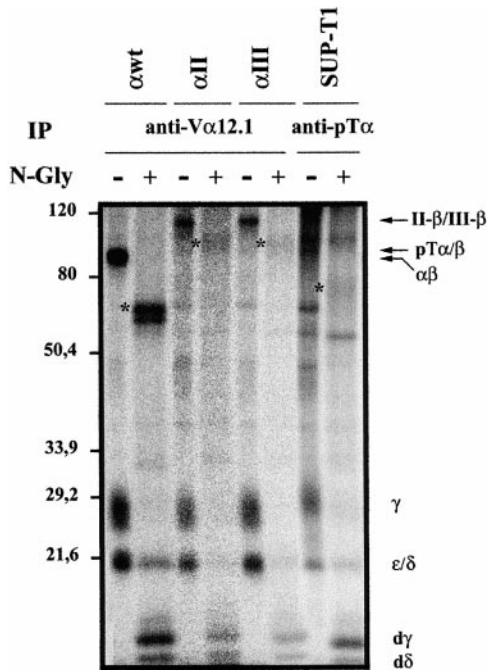


Figure 3. Biochemical composition of pre-TCR and chimeric TCR complexes expressed on SUP-T1 transfectants. Surface 125 I-labeled SUP-T1 pre-T cells either mock transfected (SUP-T1) or transfected with wild-type TCR- α (α wt) or with α II and α III TCR- α -pT α chimeric chains were lysed in 1% Brij 96-containing buffer, and the surface receptors were immunoprecipitated with an anti-V $\alpha_{12.1}$ mAb or with a rabbit antiserum against the pT α CY domain. Immunoprecipitates (10^7 cell equivalents per lane) were either digested with N-Gly (+) or left untreated (-), and resolved by 12% SDS-PAGE under nonreducing conditions. The positions of the pT α -TCR- β , TCR- α/β , α II-TCR- β and α III-TCR- β heterodimers, CD3 $\delta\epsilon$, and CD3 γ chains, and deglycosylated CD3 γ ($d\gamma$) and CD3 δ ($d\delta$) are indicated on the right. The migration positions of the molecular weight standards (in kD) are indicated on the left. *, N-deglycosylated heterodimers.

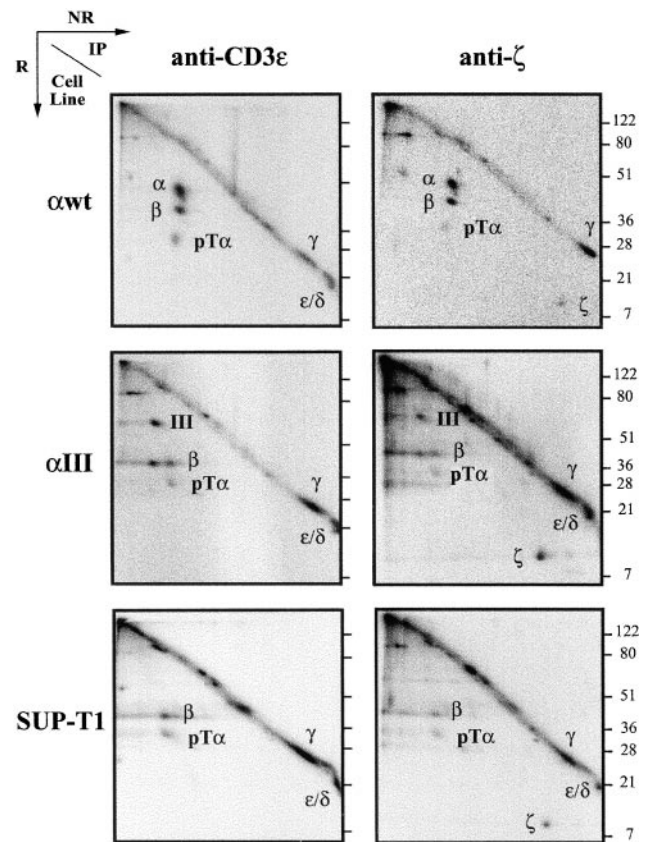


Figure 4. The ζ chain is physically associated to the pre-TCR as well as to the α III chimeric TCR. Surface iodinated SUP-T1 cells either mock transfected (SUP-T1) or transfected with the α III TCR- α -pT α chimera (α III) or with the wild-type TCR α chain were lysed in 1% Brij 96-containing buffer and surface receptors were then immunoprecipitated with a rabbit anti-human ζ chain antiserum or with an anti-CD3 ϵ mAb. The immunoprecipitates (IP) were resolved by two-dimensional nonreducing (NR)/reducing (R) SDS-PAGE. The positions of the individual pre-TCR or TCR components migrating out of the diagonal are shown, and the relative positions of the molecular weight standards are indicated on the right.

reflecting poor ζ chain iodination due to its short EC domain (33).

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

The Cyto Tail of pT α 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/ ζ components (although quantitative differences can not be ruled out), we next investigated whether the pT α Cyto tail by itself could be directly responsible for controlling surface expression levels of the whole receptor complex. To test

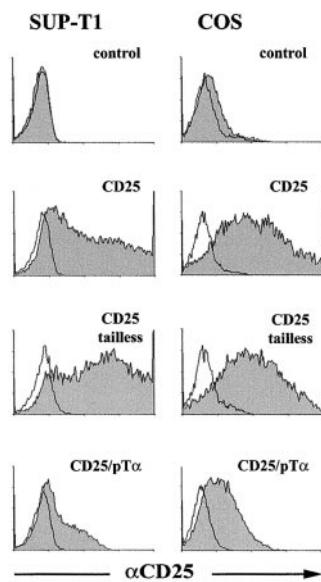


Figure 5. Impaired surface expression of a CD25-pT α chimeric protein bearing the human pT α Cyto tail. Relative levels of surface expression of murine CD25 were analyzed by flow cytometry (shaded histograms) with a biotin-coupled anti-CD25 mAb plus PE-coupled streptavidin on SUP-T1 pre-T cells or COS 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 pT α Cyto tail (CD25/pT α). 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.

this possibility, we first analyzed the effect of the pT α Cyto domain on the surface expression levels of individual plasma membrane reporter proteins. Murine CD25 (IL-2 receptor α 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 pT α results in a >50-fold reduction of surface CD25 expression on COS cells transfected with the CD25-pT α 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-pT α chimera (Fig. 5). In both cell types, the relative levels of CD25 expression were not

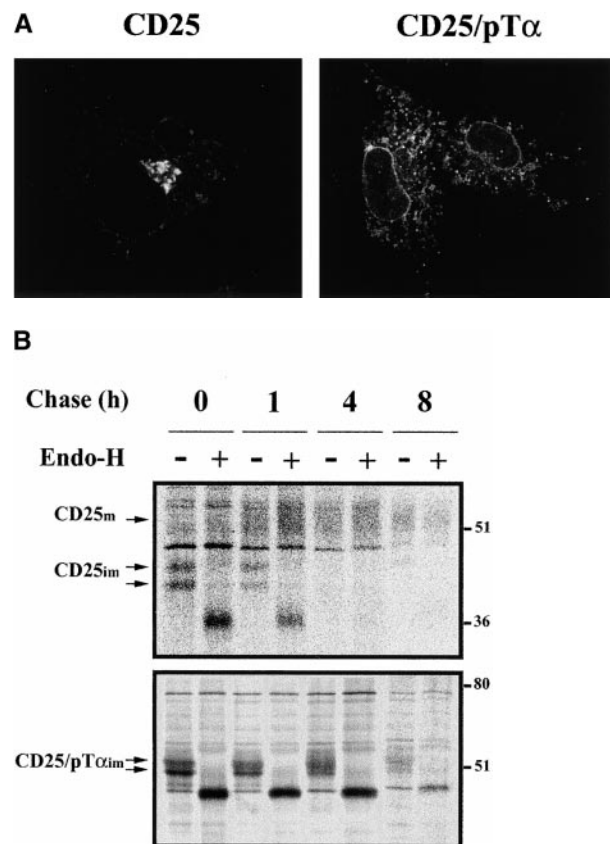


Figure 6. ER retention of a CD25-pT α chimeric protein bearing the Cyto tail of human pT α . (A) COS cells were transfected with either cDNA encoding the wild-type murine CD25 or with a CD25/pT α chimeric cDNA engineered to encode the CD25 EC and TM domains and the pT α Cyto tail (CD25/pT α). 48 h after 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-pT α COS transfectants were pulse-chase labeled with [35 S]methionine/cysteine and lysed in Triton X-100-containing lysis buffer. CD25 (top) and CD25-pT α (bottom) immunoprecipitates obtained with an anti-mouse CD25 (PC.61) mAb or with a rabbit antiserum against the human pT α 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 pT α Cyto tail.

To assess whether the CD25-pT α 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 tubular-vesicular peripheral staining was observed in COS cells transfected with the CD25-pT α chimera, which strongly suggested an ER distribution. To confirm that the pT α tail was in fact conferring ER residence to membrane proteins, we next examined susceptibility to endo-H digestion of wild-type and chimeric CD25-pT α 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 pT α 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-pT α chimeras analyzed from either anti-pT α (Fig. 6 B, bottom) or anti-CD25 precipitates (data not shown), as none of them ac-

quire 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-pT α 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-pT α 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 pT α 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 pT α 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-pT α Ab CT-1 in combination with fluorescein-conjugated goat anti-rabbit Igs, native pT α 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 pT α -GFP chimeric protein (Fig. 7). To provide formal proof that the intracellular compartment containing pT α was in fact the ER, double-color analyses were performed in pT α -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 pT α -GFP transfectants. Moreover, costaining with an anti-TCR- β (β F1) mAb followed by Cy5-conjugated goat anti-mouse Igs revealed that endogenous TCR- β colocal-

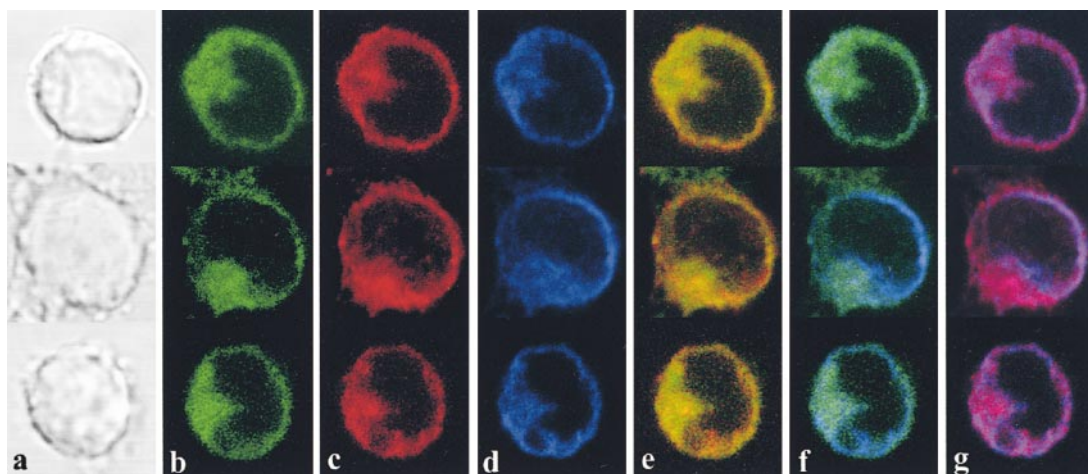


Figure 7. Colocalization of pT α -GFP with the ER-resident protein PDI. SUP-T1 cells stably transfected with pT α -GFP were fixed, permeabilized, and stained with an anti-TCR- β mAb followed by Cy5-coupled goat anti-mouse Igs, and an anti-PDI rabbit antiserum followed by Cy3-coupled goat anti-rabbit Igs. Analysis of pT α -GFP and TCR- β intracellular localization was performed by confocal microscopy. (a) Nomarsky analysis of selected individual cells. (b) Expression pattern of the pT α -GFP chain. (c) Intracellular staining of the ER-resident protein PDI. (d) Intracellular TCR- β chain staining. (e-g) Overlays of pT α -GFP and PDI (e), pT α -GFP and TCR- β (f), and TCR- β and PDI (g) stainings. The panels of individual cells shown are representative of all SUP-T1 cells examined. Original magnification: $\times 63$.

ized with GFP and PDI. Thus, from these experiments we concluded that intracellular pT α and TCR- β are predominantly retained in the ER in SUP-T1 pre-T cells.

Partial Truncation of the pT α Cyto Tail Releases a CD4-pT α Chimeric Protein from ER Retention and Restores Cell Surface Protein Expression. Collectively, the above experiments provided evidence that the Cyto domain of pT α is directly responsible for retaining proteins in the ER. Thus, it was reasonable to predict that deletion of the pT α 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 pT α tail deletion was the opposite, as it prevented surface pre-TCR expression. Indeed, JR3.11 cells transfected with a tailless pT α chain failed to react with anti-CD3 mAbs (Fig. 8 A), as well as with an anti-TCR- β (V β 8) mAb and an anti-pT α Ab (data not shown). This unexpected discrepancy could be explained if the cytoplasmic domain of pT α 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 pT α tail prompted us to define more precisely the location of the potential ER retention signal in the pT α Cyto domain. To this end, we examined the effect of successive truncations along the pT α tail on the intracellular location and surface expression levels of individual CD4-pT α 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-pT α chimeric constructs where the Cyto domain of human CD4 was replaced with the full pT α tail (CD4-pT α) revealed a clear tubular-vesicular staining of the ER that was virtually identical to that recorded for the CD25-pT α 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 pT α residues by a truncation at position 243 (CD4-pT α 22), although this truncation resulted in a partial increase of surface CD4 expression on the corresponding BW transfected cells (Fig. 8 B). In contrast, when an additional 26 amino acids (aa) were removed, the resulting CD4-pT α 48 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 pT α Cyto domain between aa 217 and 243 is responsible for the observed ER retention function of the pT α tail. Accordingly, the CD4-pT α 48 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

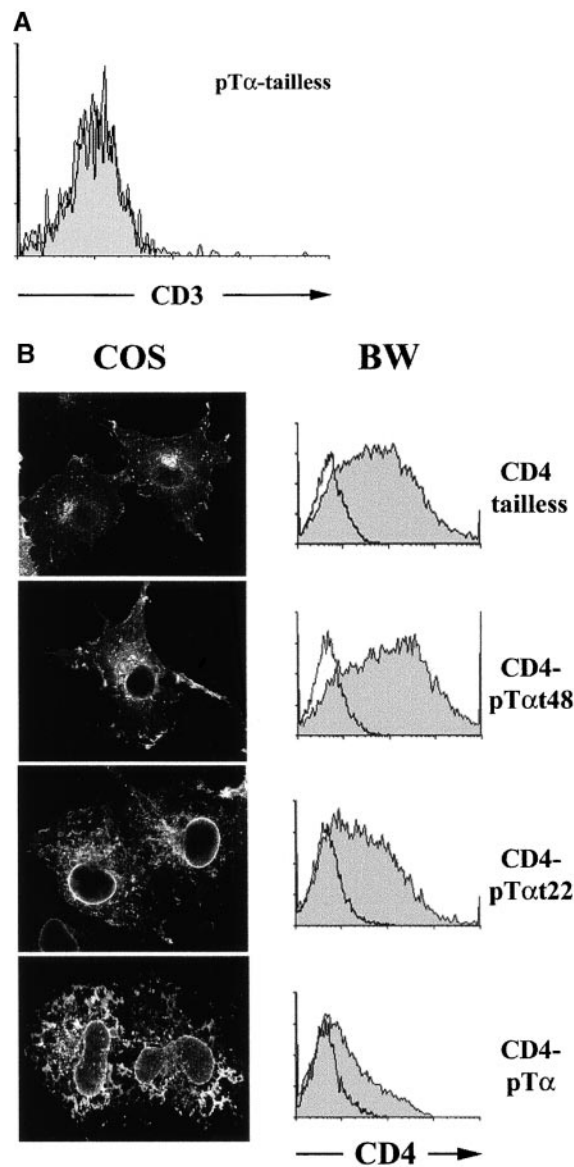


Figure 8. pT α tailless molecules fail to reach the plasma membrane, but a partial truncation of the pT α Cyto tail releases a CD4-pT α chimeric protein from ER retention and restores cell surface expression. (A) JR3.11 cells transfected with a bicistronic vector encoding GFP and a pT α tailless molecule were stained with a PE-coupled anti-CD3 mAb. Flow cytometry analysis of surface CD3 expression (shaded histogram) was performed 48 h after transfection on electronically gated GFP⁺ cells. Background values (unshaded histogram) were obtained with a PE-coupled isotype-matched irrelevant mAb. (B) COS and BW cells were transfected with cDNAs encoding human CD4 molecules in which the Cyto domain has been deleted (CD4 tailless) or replaced either with a full pT α Cyto domain (CD4-pT α) or with a truncated pT α tail lacking the COOH-terminal 22 (CD4-pT α 22) or 48 (CD4-pT α 48) residues. 48 h after transfection, COS cells were fixed, permeabilized, and stained with an anti-CD4 mAb plus FITC-coupled goat anti-mouse Igs, and then analyzed by immunofluorescence microscopy (original magnification: $\times 63$). BW cells were stained 24 h after transfection with a Cy5-PE-coupled anti-CD4 mAb (shaded histograms) and analyzed by flow cytometry. Control values (unshaded histograms) were obtained with a Cy5-PE-coupled isotype-matched irrelevant mAb.

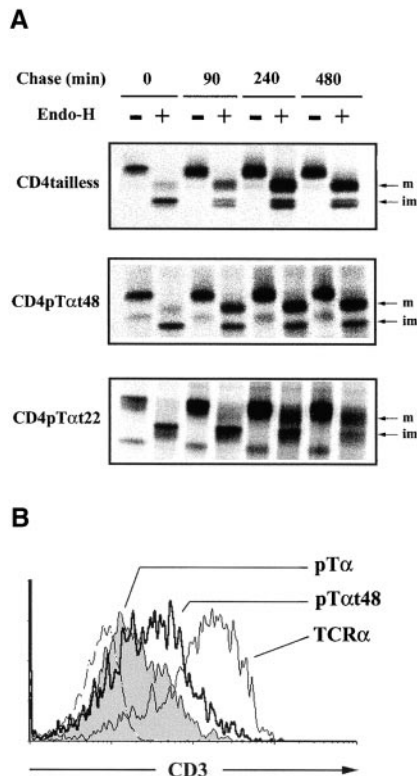


Figure 9. Truncation of the COOH-terminal 48 aa of the pT α Cyto domain restores transport out of the ER of a CD4-pT α chimeric protein and correlates with enhanced expression of the pre-TCR (A) COS cells transfected with the CD4 tailless, CD4-pT α 48, or CD4-pT α 22 constructs described in the legend to Fig. 8 were pulse-chased labeled and lysed as described in the legend to Fig. 6. Anti-CD4 immunoprecipitates were treated with endo-H (+) or left untreated (-) and resolved by 12% SDS-PAGE under reducing conditions. (B) CD3 surface expression was analyzed by flow cytometry, as described in the legend to Fig. 8 A, on JR3.11 cells transiently transfected with bicistronic vectors encoding GFP and either the wild-type pT α (shaded histogram) or a truncated pT α lacking the COOH-terminal 48 residues (pT α 48; bold line), or a wild-type TCR- α (thin line). Background values (dotted line) were obtained with a PE-coupled isotype-matched irrelevant mAb. The positions of the mature (m) and immature (im) proteins is indicated on the right.

shown in Fig. 9 A, both proteins acquired endo-H resistance after a 90-min chase, and were mostly converted after 4 h. In contrast, the CD4-pT α 22 chimera remained essentially endo-H sensitive after 90 min and up to 50% of pulsed chimeric protein failed to acquire endo-H resistance, even after an 8-h chase.

Finally, experiments were performed to analyze the effect of equivalent truncations along the pT α protein on pre-TCR surface expression. Bicistronic vectors containing such pT α constructs allowed tracing of transiently transfected JR3.11 cells by EGFP expression as a separate but not as a chimeric protein. As shown in Fig. 9 B, although the pT α deletions were not sufficient to reach TCR- α expression levels, JR3.11 cells transfected with a pT α chain lacking the terminal 48 residues (pT α 48) consistently showed a modest increase of surface CD3 levels, compared with CD3 expression on wild-type pT α transfectants. Thus, as observed with the CD4-pT α 48 chimera, this

particular truncation may release pT α from ER retention, resulting in enhanced surface pre-TCR expression. In contrast, surface expression of a pre-TCR composed of the pT α 22 truncated protein was indistinguishable from expression levels of a wild-type pre-TCR. Collectively, our data suggest that the observed pT α 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 pT α 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 -pT α 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 pT α are not competing each other for pairing with TCR- β . Conversely, transfection of a TCR- α -deficient mature T cell line with a pT α 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 pT α chain itself might play a direct role in controlling pre-TCR expression levels.

In an effort to identify the structural domain/s of pT α responsible for such a role, we took advantage of the similarities and differences between the EC, TM, and Cyto domains of pT α and TCR- α and produced TCR- α -pT α 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 pT α region, which lacks a covalently associated Ig-like V domain (25), and asked the question of whether replacement of the pT α EC domain with the EC region of TCR- α could provide a symmetrical shape to the TCR- β -pT α heterodimer that could result in an increased expression at the cell surface. Unexpectedly, however, such chimeric TCR- α -pT α chains (α II) did not affect the levels of expression of the pre-TCR, indicating that low surface pre-TCR expression is not dependent on the pT α EC domain. From these data one could infer that the lack in pT α of a partner domain capable of pairing with the hydrophobic surface of the V β 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

transport of the TCR- β -pT α heterodimer to the cell surface (2, 6). Expression of such a putative VpreT is expected to be developmentally regulated, such that its absence would account for the impaired surface pre-TCR expression described in murine mature T cells (6). However, human mature T cells proved to be as efficient as pre-T cells in expressing a functional TCR- β -pT α heterodimer on their surface (this study and unpublished results), thus supporting the alternative hypothesis that the pT α chain is sufficient 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 pT α EC domain was replaced with the EC TCR- α domain behaves as a wild-type pT α chain in terms of surface receptor expression was not obvious, because the TCR- α C domain displays little homology with the equivalent pT α domain (9, 25). However, as the TCR- α C domain residues involved in polar interactions with the TCR- β C domain are all conserved in the pT α C domain, it has been proposed that association between pT α and TCR- β C domains may occur in a way similar to that observed in the TCR C α C β module (3, 37). These data are against an essential function of TCR- β -pT α assembly in controlling surface expression levels of the pre-TCR, and suggest that the CD3/ ζ subunits associated with the TCR- β -pT α 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 stability (19–22, 24), and CD3 δ seems dispensable for pre-TCR assembly and function (8, 13, 38). In contrast, the CD3 γ ϵ 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 pT α 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 CD3 γ , CD3 δ , and CD3 ϵ subunits. What is more surprising is that all the wild-type pre-TCR and the chimeric TCR complexes 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 connecting peptide (CP) of pT α 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 results allowed us to conclude that qualitative differences in the contribution of the CD3/ ζ components to pre-TCR or TCR assembly do not influence surface receptor expression levels.

A striking finding was that replacing the Cyto domain of TCR- α with the equivalent pT α domain is sufficient for inducing low surface expression of the chimeric TCR. It is thus obvious that the pT α Cyto tail is directly responsible

for low surface pre-TCR expression. A strong argument in favor of the attractive possibility that the pT α Cyto tail functions as an ER retention signal was the observation that individual plasma membrane proteins are mostly retained in the ER and fail to reach the Golgi compartment when appended to the pT α tail. Accordingly, pT α chains are primarily found in internal structures which colocalize with the ER. The demonstration that truncation of the terminal 48 aa of pT α (and particularly deletion of the region between residues 217 and 243) releases CD4-pT α chimeric proteins from ER retention lends further support to that notion, and suggests that pT α ER retention determinants localize primarily in the deleted region. However, the pT α tail sequence from position 217 to 243 does not harbor the consensus dilysine cytoplasmic motif for ER retention of TM proteins (34) or the reported CD3 ϵ ER retention sequence (44), suggesting the existence of alternative ER retention signals. An extensive series of point mutations will have to be introduced into that particular pT α 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 pT α Cyto tail that is essential for surface expression of the pre-TCR, as pT α tailless molecules fail to bring a CD3-associated pre-TCR complex to the cell surface. Therefore, efficient surface expression of the pre-TCR complex may be the result of a balance between complex mechanisms controlling retention and assembly of its individual components. This may explain the finding that release of individual chimeras bearing a truncated pT α tail from ER retention correlates with recovery of wild-type protein expression levels, whereas pT α molecules with identical truncations were unable to recover TCR- α / β surface levels, although they were more efficient than wild-type pT α 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., pT α and λ 5) 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 conservation of the Cyto tail in both species (6, 25) would make this possibility unlikely. However, conserved residues in the cytoplasmic domain have been identified (25). In addition, two independent findings strengthen our view: first, DN thymocytes from pT α ^{-/-} mice made transgenic for a pT α tailless chain display increased TCR- β surface levels, compared with DN thymocytes from wild-type or pT α ^{-/-} nontransgenic mice (46), and second, low CD3 expression is common to DP thy-

mocytes from recombination activating gene (Rag)1^{-/-} mice expressing a transgenic functional pre-TCR consisting of truncated TCR- β and pT α forms that keep their TM and Cyto regions (14). In conclusion, our data provide evidence that the pT α 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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