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Developmental regulation of αβ T cell antigen receptor expression results from differential stability of nascent TCRα proteins within the endoplasmic reticulum of immature and mature T cells

Kelly P.Kearse[2], Joseph L.Roberts, Terry I.Munitz, David L.Wiest, Toshinori Nakayama[1] and Alfred Singer

Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
[1]Present address: Department of Immunology, Faculty of Medicine, University of Tokyo, Tokyo, Japan
[2]Corresponding author

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The αβ T cell antigen receptor (TCR) that is expressed on most T lymphocytes is a multisubunit transmembrane complex composed of at least six different proteins (α, β, γ, δ, ε and ζ) that are assembled in the endoplasmic reticulum (ER) and then transported to the plasma membrane. Expression of the TCR complex is quantitatively regulated during T cell development, with immature $CD4^+CD8^+$ thymocytes expressing only 10% of the number of surface αβ TCR complexes that are expressed on mature T cells. However, the molecular basis for low TCR expression in developing αβ T cells is unknown. In the present study we report the unexpected finding that assembly of nascent component chains into complete TCRαβ complexes is severely impaired in immature $CD4^+CD8^+$ thymocytes relative to their mature T cell progeny. In particular, the initial association of TCRα with TCRβ proteins, which occurs relatively efficiently in mature T cells, is markedly inefficient in immature $CD4^+CD8^+$ thymocytes, even for a matched pair of transgenic TCRα and TCRβ proteins. Inefficient formation of TCRαβ heterodimers in immature $CD4^+CD8^+$ thymocytes was found to result from the unique instability of nascent TCRα proteins within the ER of immature $CD4^+CD8^+$ thymocytes, with nascent TCRα proteins having a median survival time of only 15 min in $CD4^+CD8^+$ thymocytes, but >75 min in mature T cells. Thus, these data demonstrate that stability of TCRα proteins within the ER is developmentally regulated and provide a molecular basis for quantitative differences in αβ TCR expression on immature and mature T cells. In addition, these results provide the first example of a receptor complex whose expression is quantitatively regulated during development by post-translational limitations on receptor assembly.

Key words: allelic exclusion/T cell differentiation/T cell receptor

Introduction

Differentiation of immature precursor cells into mature αβ T cells occurs in the thymus via a series of ordered steps that are best characterized by variable expression of CD4 and CD8 co-receptor molecules (Fowlkes and Pardoll, 1989). The earliest precursor cells in the thymus express neither CD4 nor CD8 co-receptor molecules and are therefore $CD4^-CD8^-$. Productive rearrangement of their TCRβ gene locus induces $CD4^-CD8^-$ precursor cells to differentiate into $CD4^+CD8^+$ thymocytes, during which they rearrange their T cell antigen receptor (TCR) α gene locus and begin to assemble and express low levels of αβ TCR complexes on their cell surfaces (Takahama *et al.*, 1992). The developmental fate of individual $CD4^+CD8^+$ thymocytes is ultimately determined by the specificity of the αβ TCR complex each expresses, with only $CD4^+CD8^+$ thymocytes expressing TCR of appropriate specificities being positively selected for further differentiation into mature $CD4^+$ or $CD8^+$ T cells (Kieslow *et al.*, 1988; Blackman *et al.*, 1990).

Because the developmental fate of immature $CD4^+CD8^+$ thymocytes is determined by the specificity of their αβ TCR, it is curious that immature $CD4^+CD8^+$ thymocytes express only 10% of the number of αβ TCR complexes that are expressed by mature T cells (Finkel *et al.*, 1987; Havran *et al.*, 1987). Expression of αβ TCR complexes by $CD4^+CD8^+$ thymocytes increases during positive selection, indicating that expression of αβ TCR complexes is quantitatively regulated during differentiation and selection in the thymus. However, it is not known how such quantitative regulation of αβ TCR expression is accomplished. Despite marked differences in the number of complete αβ TCR complexes expressed by immature thymocytes versus mature T cells, TCR expression does not appear to be quantitatively regulated during development by transcriptional mechanisms, as immature thymocytes and mature T cells contain approximately equivalent amounts of mRNA encoding component TCR proteins (Maguire *et al.*, 1990). While synthesis of individual TCR components occurs at a relatively high rate (Bonifacino *et al.*, 1990a; Kosugi *et al.*, 1992), immature $CD4^+CD8^+$ thymocytes degrade a large percentage of most TCR components that they synthesize within the endoplasmic reticulum (ER), suggesting that αβ TCR expression is post-translationally regulated during development (Bonifacino *et al.*, 1990a). However, the molecular basis for quantitative regulation of αβ TCR expression in immature $CD4^+CD8^+$ thymocytes has not been elucidated. Two explanations have previously been put forward: (i) complete αβ TCR complexes that are competent to exit the ER of mature T cells are unable to escape from the ER of immature thymocytes, resulting in their disassembly and degradation (Bonifacino *et al.*, 1990a); or (ii) the number of complete TCR complexes assembled in $CD4^+CD8^+$ thymocytes is severely limiting because of a cryptic assembly defect (Kearse *et al.*, 1993).

Understanding the molecular basis for low expression of αβ TCR complexes in immature thymocytes is central

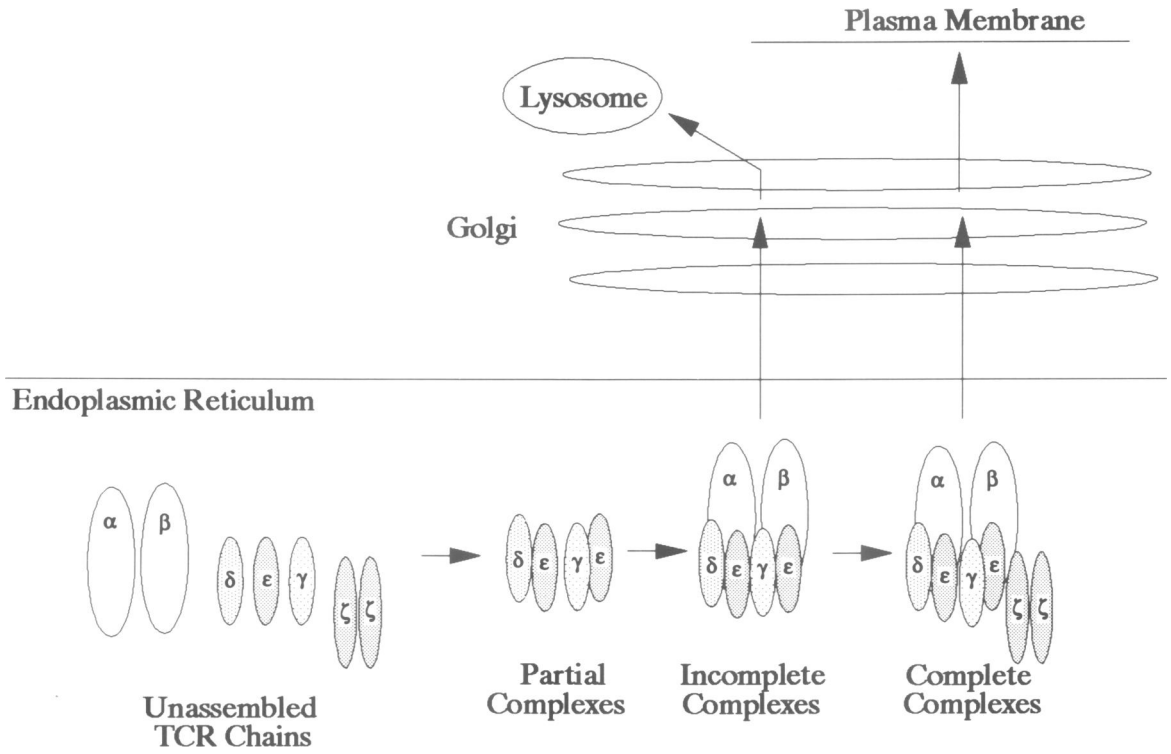

Fig. 1. Assembly and intracellular transport of murine TCR proteins. Model illustrating assembly of TCR components into partial, incomplete and complete forms of the TCR. Unassembled individual TCR chains and partial complexes of CD3 components are retained within the ER. Whereas incomplete (αβγδε) and complete (αβγδεζ) forms of the TCR complex are competent to exit the ER, only complete TCR complexes are efficiently transported to the plasma membrane. The reader is referred to the text for details.

to understanding the molecular basis of positive selection signals that increase it, as well as being important for understanding the physiology of $CD4^+CD8^+$ thymocytes, which constitute the majority of cells in the thymus. Consequently, the present study has examined the biochemical basis for low αβ TCR expression in immature $CD4^+CD8^+$ thymocytes. Contrary to previous suggestions, complete αβ TCR complexes were found to be stable and to efficiently exit the ER of immature $CD4^+CD8^+$ thymocytes. Surprisingly, we found that immature $CD4^+CD8^+$ thymocytes were intrinsically less able than their mature T cell progeny to assemble component TCR chains into complete TCRαβ complexes. A limiting point in TCR assembly in immature $CD4^+CD8^+$ thymocytes was found to be the initial association of nascent TCRα with TCRβ proteins, which occurs at a low rate in immature $CD4^+CD8^+$ thymocytes because of an exceptionally rapid rate at which nascent TCRα proteins are degraded. Thus, the present study demonstrates that assembly of multisubunit αβ TCR complexes from individual component chains is quantitatively regulated in immature T cells, providing the first example of a receptor complex whose expression is quantitatively regulated during development by post-translational limitations on receptor assembly.

Results

Assembly of αβ TCR complexes.

αβ TCR complexes are composed of three protein families: clonotypic TCRαβ chains, CD3 chains (CD3γ, δ and ε) and TCRζ chains (Klausner *et al.*, 1990), (Figure 1). Assembly of murine αβ TCR complexes from individual component chains occurs in the ER by a series of ordered steps involving: (i) assembly of CD3 proteins into partial complexes of CD3 components; (ii) association of αβ proteins with CD3 components to form αβγδε incomplete complexes; (iii) association of αβγδε complexes with disulfide-linked TCRζ chain homodimers to form complete αβγδεζ complexes that exit the ER and that are ultimately transported to the cell surface (Ohashi *et al.*, 1985; Saito *et al.*, 1987; Alarcon *et al.*, 1988; Bonifacino *et al.*, 1988). CD3 and TCRαβ proteins that fail to be assembled into larger complexes are retained within the ER and, depending upon the particular protein, degraded (Minami *et al.*, 1987; Chen *et al.*, 1988; Bonifacino *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989a; Wileman *et al.*, 1990). Incomplete αβγδε complexes that do not associate with TCRζ can exit the ER, but are targeted to lysosomes for degradation (Sussman *et al.*, 1988). Only complete αβγδεζ TCR complexes are efficiently transported to the cell surface (Sussman *et al.*, 1988; Hall *et al.*, 1991).

Fully assembled TCR complexes exit the ER of immature $CD4^+CD8^+$ thymocytes

It has been suggested that the high rate of degradation of some nascent TCR chains within the ER of $CD4^+CD8^+$ thymocytes may reflect the failure of complete TCR complexes to exit the ER (Bonifacino *et al.*, 1990a); alternatively, it may reflect the existence of a cryptic assembly defect in $CD4^+CD8^+$ thymocytes that limits the assembly of TCR components into complete TCR complexes, resulting in partial TCR complexes that are incompetent to exit the ER (Kearse *et al.*, 1993). To evaluate these issues, we examined the assembly and

intracellular transport of TCR complexes in immature $CD4^+CD8^+$ thymocytes and mature splenic T cells.

First, we examined whether newly assembled complete TCR complexes exit the ER of immature $CD4^+CD8^+$ thymocytes. The lectin *Ricinus communis* agglutinin (RCA) can distinguish TCR complexes present in the ER from those which have reached at least the *trans*-Golgi compartment of the cell, because it specifically binds oligosaccharide side chains on TCR glycoproteins that contain either terminal galactose residues or internal galactose residues capped by sialic acid, both of which are added in the *trans*-Golgi compartment of the cell (Lotan *et al.*, 1977; Kornfeld and Kornfeld, 1985; Mellman and Simons, 1992) (see Figure 2A). In these experiments purified $CD4^+CD8^+$ thymocytes were metabolically labeled with [^{35}S]methionine and chased for 2.5 h to allow movement of metabolically labeled proteins from the ER to the Golgi apparatus. Cells were lysed in 1% digitonin and the lysates were either unfractionated or exposed to RCA matrices and separated into RCA-unbound and RCA-bound fractions; RCA-bound material was subsequently eluted from the RCA by addition of 200 mM β-lactose. TCR complexes present in unfractionated material, RCA-unbound and RCA-bound fractions were immunoprecipitated with monoclonal antibodies (mAb) to CD3ε and subjected to digestion with the enzyme endoglycosidase H (Endo H), which is specific for N-linked oligosaccharides that have not been processed by Golgi glycosidases and glycosyltransferases (Tarentino and Maley, 1974). RCA fractionation effectively separated immature and mature TCR complexes, as Endo H-sensitive TCR chains were only present in RCA-unbound fractions and Endo H-resistant TCR chains were only present in RCA-bound fractions (Figure 2B).

Most importantly, the number of TCRζ chains immunoprecipitated by mAb to CD3ε is a direct measure of the number of fully assembled TCR complexes (Figure 1); for quantitative purposes the relative number of TCRζ chains assembled with CD3ε during the pulse period was set at 1.0 (Figure 2C). Of the number of complete TCR complexes assembled during the 30 min pulse period, 50% were present in the RCA-unbound pulse fraction and 50% were already present in the RCA-bound pulse fraction (Figure 2C). After 150 min of chase, more than 95% of newly assembled TCRζ chains were found in the RCA-bound chase fraction (Figure 1, bottom), indicating that essentially all newly assembled TCR complexes that were originally present in the RCA-unbound pulse fraction were subsequently recovered in the RCA-bound chase fraction.

Thus, these experiments provide direct evidence that newly assembled complete TCR complexes rapidly exited the ER of $CD4^+CD8^+$ thymocytes and transited to at least the *trans*-Golgi compartment of the cell. This result effectively excludes the possibility that low surface expression of TCR on $CD4^+CD8^+$ thymocytes results from the retention of complete TCR complexes within the ER.

Formation of complete TCR complexes is impaired in $CD4^+CD8^+$ thymocytes

Because complete TCR complexes efficiently exited the ER of $CD4^+CD8^+$ thymocytes, we next compared immature $CD4^+CD8^+$ thymocytes and mature splenic T cells for their ability to assemble complete TCR complexes

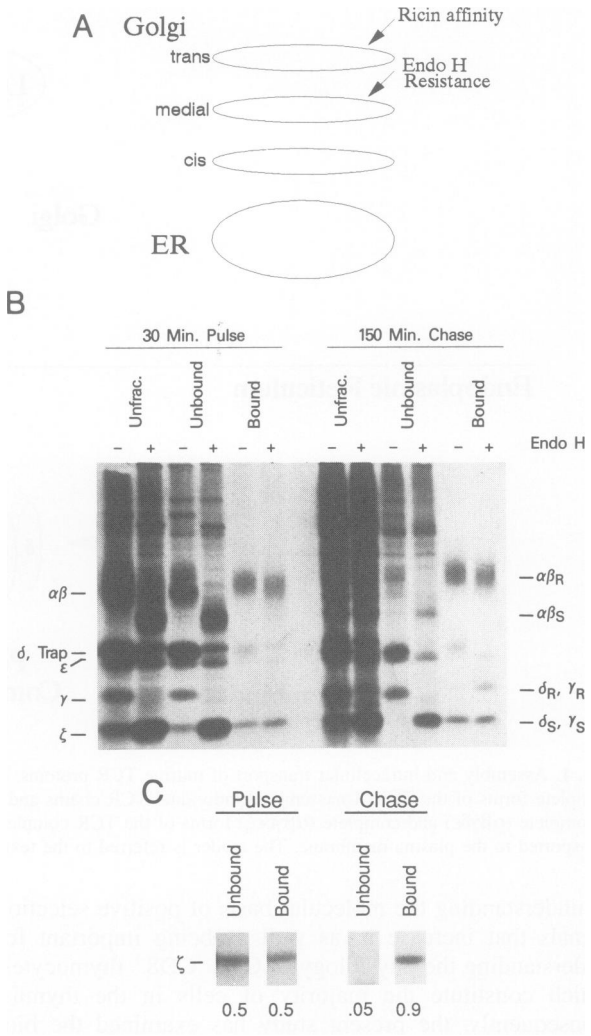

Fig. 2. Newly formed complete TCR rapidly exit the ER in $CD4^+CD8^+$ thymocytes. (**A**) Separation of immature and mature TCR complexes in $CD4^+CD8^+$ thymocytes via lectin affinity chromatography. (**B**) Radiolabeled lysates of $CD4^+CD8^+$ thymocytes were either unfractionated or passed over a *Ricinus communis* agglutinin (RCA) matrix to separate immature and mature TCR complexes. Unfractionated and RCA-fractionated material was precipitated with mAb to CD3ε, mock treated or digested with Endo H and analyzed by one-dimensional SDS–PAGE under reducing conditions. The positions of α, β, γ, δ, TRAP (TCR associated protein), ε and ζ chains are indicated. The mobilities of immature, Endo H-sensitive ($\alpha\beta_S$, δ_S and γ_S) and mature, Endo H-resistant ($\alpha\beta_R$, δ_R and γ_R) TCR glycoproteins are indicated. (**C**) The relative number of chains assembled with CD3ε during the pulse period was set at 1.0. The amounts of CD3-associated TCRζ protein present in unbound and bound fractions of material were determined by densitometric scanning and are expressed as a fraction of newly assembled TCRζ proteins. Multiple autoradiographs were scanned to ensure linearity.

from individual component chains. We focused on the fate of newly synthesized TCRζ chains in immature thymocytes and mature T cells, as addition of TCRζ chains is the final step in assembly of complete TCR complexes and is required for surface expression of TCR (Figure 1). In these experiments the total amount of TCRζ synthesized during a 30 min pulse period was determined by immunoprecipitating metabolically labeled cell lysates with antibodies (Ab) to TCRζ (Figure 3, lanes 9–12). TCRζ chains that were assembled into complete TCR complexes were immunoprecipitated with mAb to CD3ε

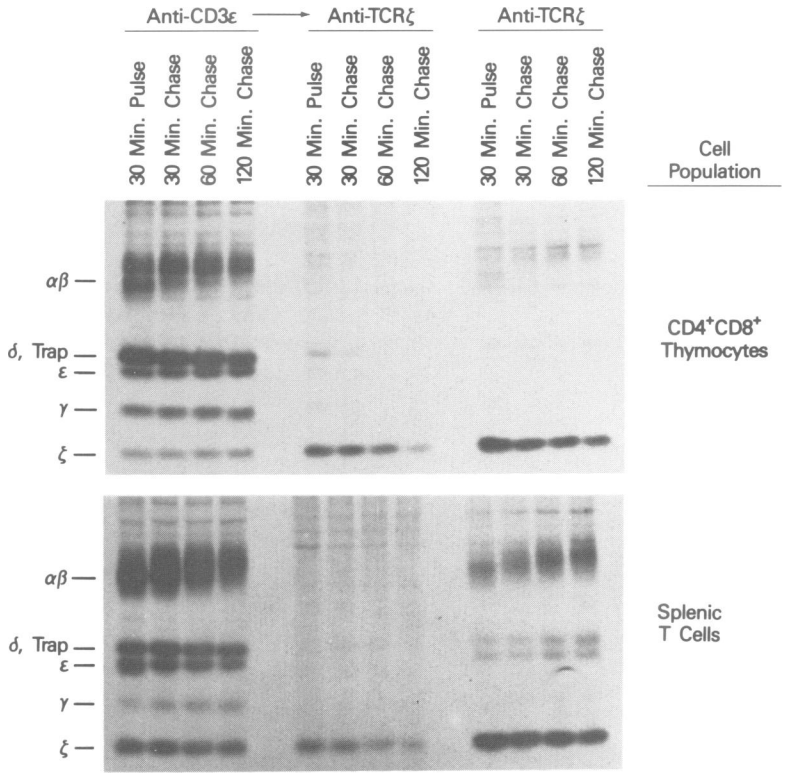

Fig. 3. The formation of complete TCR is severely limiting in $CD4^+CD8^+$ thymocytes. Purified $CD4^+CD8^+$ thymocytes and splenic T cells were labelled in methionine-free medium containing [^{5}S]methionine at 1 mCi/ml for 30 min at 37°C and chased in complete medium containing excess cold methionine for the indicated time period. Cells were solubilized in 1% digitonin and lysates sequentially immunoprecipitated with mAb to CD3ε immobilized on protein A beads, followed by mAb to TCRζ immobilized on protein A beads. Alternatively, lysates were precipitated only with mAb to TCRζ (far right-hand lanes). Immunoprecipitated material was resolved by SDS−PAGE under reducing conditions, and gels were processed for autoradiography, dried and exposed to film. The positions of TRAP, CD3γ, δ and ε and TCRα, β and ζ proteins are indicated.

(Figure 3, lanes 1–4), leaving behind unassembled TCRζ chains that could then be captured by sequentially immunoprecipitating with Ab to TCRζ (Figure 3, lanes 5–8).

In mature splenic T cells, most (67%) nascent TCRζ chains synthesized during the 30 min pulse period were associated with CD3ε, as they were immunoprecipitated with mAb to CD3ε (Figure 3, bottom, compare lane 9 with lanes 1 and 5). In contrast, only a minority (24%) of TCRζ chains that were synthesized in immature $CD4^+CD8^+$ thymocytes were immunoprecipitated by mAb to CD3ε, with most nascent chains in $CD4^+CD8^+$ thymocytes remaining unassembled (Figure 3, top, compare lane 9 with lanes 1 and 5). Thus, the failure of most newly made TCRζ chains to be immunoprecipitated by mAb to CD3ε demonstrates that assembly of complete TCR complexes in $CD4^+CD8^+$ thymocytes is significantly impaired relative to mature splenic T cells.

Despite their low number, TCR complexes that were successfully assembled within immature $CD4^+CD8^+$ thymocytes during the 30 min pulse period were stable, as the amount of ζ immunoprecipitated by mAb to CD3ε after the pulse period remained unchanged throughout the chase period (Figure 3, lanes 1–4). In contrast to assembled ζ proteins, unassembled ζ proteins were degraded in both immature thymocytes and mature T cells within 120 min of their synthesis (Figure 3, lanes 5–8).

Thus, assembly of TCR chains into complete αβγδεζ TCR complexes is markedly less efficient in $CD4^+CD8^+$ thymocytes than in mature T cells, but TCR complexes that are assembled in $CD4^+CD8^+$ thymocytes are stable and are not disassembled. These data indicate that TCR assembly is impaired in $CD4^+CD8^+$ thymocytes and that the impairment was proximal to the addition of TCRζ chains.

Impaired formation of αβγδε TCR intermediates in $CD4^+CD8^+$ thymocytes

As can also be appreciated from Figure 3, the relative amounts of nascent TCRαβ proteins that assembled with CD3 components were several-fold lower in immature $CD4^+CD8^+$ thymocytes than in splenic T cells (Figure 3, compare upper and lower panels, lanes 1–4), indicating that immature $CD4^+CD8^+$ thymocytes were inefficient in assembling αβγδε TCR intermediates. To confirm that the assembly step that is impaired in $CD4^+CD8^+$ thymocytes was proximal to the assembly of TCRαβ proteins with CD3 components into αβγδε intermediates, we examined the distribution of CD3δ chains among TCR complexes at different stages of assembly in immature thymocytes and mature T cells. Cell lysates were sequentially immunoprecipitated with: (i) Ab to TCRζ to precipitate CD3δ chains assembled into complete αβγδεζ TCR complexes; (ii) mAb to TCRβ to precipitate CD3δ proteins assembled into incomplete αβγδε TCR complexes; (iii) mAb to CD3ε to precipitate CD3δ chains assembled into partial γδε complexes; and, finally, (iv) Ab to CD3δ to precipitate unassembled CD3δ chains (see Figure 1). Immunoprecipitates were resolved on SDS−PAGE and immuno-

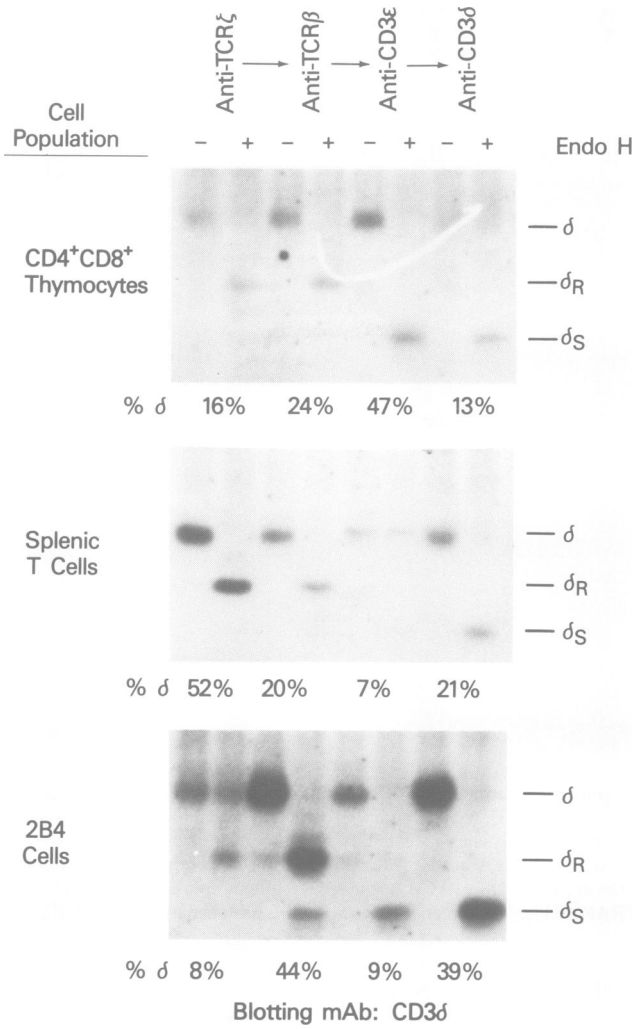

Fig. 4. Most CD3δ chains exist in partial complexes of CD3 components in $CD4^+CD8^+$ thymocytes. Digitonin lysates of $CD4^+CD8^+$ thymocytes, splenic T cells and 2B4 T hybridoma cells were sequentially immunoprecipitated with Ab to TCRζ to isolate complete (αβγδεζ) TCR complexes, followed by mAb to TCRβ to isolate incomplete (αβγδε) TCR intermediates, followed by mAb to CD3ε to isolate partial (γδε) complexes, followed by Ab to CD3δ to isolate unassembled CD3δ chains. Immunoprecipitated material was mock treated or digested with Endo H, resolved by SDS−PAGE under reducing conditions, transferred to nitrocellulose and probed with Ab to CD3δ (#R9). Immature (Endo H-sensitive, δ_S) and mature (Endo H-resistant, δ_R) forms of CD3δ are indicated. The relative amount of CD3δ proteins present in each precipitate was quantitated by densitometry and is presented as %δ in each fraction with total %δ set equal to 100%. Multiple autoradiographs of different exposures were scanned to ensure linearity.

blotted with Ab to CD3δ. In $CD4^+CD8^+$ thymocytes, most CD3δ chains were neither associated with TCRζ nor TCRβ proteins, but existed in partial complexes of CD3 components (Figure 4, top). In contrast, most CD3δ chains in splenic T cells were assembled into complete TCR complexes, as they were isolated with Ab to TCRζ (Figure 4, middle). In lysates of the T hybridoma cell line 2B4, fewer CD3δ chains were associated with TCRζ than TCRβ proteins, since TCRζ proteins are limiting in 2B4 cells (Figure 4, bottom). In both $CD4^+CD8^+$ thymocytes and splenic T cells, CD3δ chains that were present in complete TCR complexes containing TCRζ were exclusively Endo H-resistant, indicative of exit from the ER and transit to at least the medial Golgi compartment of the cell. These data indicate that formation of incomplete αβγδε TCR intermediates is impaired in $CD4^+CD8^+$ thymocytes and that most CD3δ chains are retained within the ER of $CD4^+CD8^+$ thymocytes, because they are not assembled into the minimal TCR complex (i.e. αβγδε) that is competent to exit the ER (Figure 1).

Inefficient association of TCRα and β proteins in $CD4^+CD8^+$ thymocytes

Because formation of incomplete αβγδε TCR intermediates requires association of TCRα and β proteins, we compared the association of nascent TCRα and β proteins in $CD4^+CD8^+$ thymocytes and splenic T cells. Cells were metabolically labeled with [^{35}S]methionine, lysed in 1% NP-40 and the lysates resolved by two-dimensional non-equilibrium pH electrophoresis (NEPHGE)/SDS−PAGE under reducing conditions to separate TCRα and TCRβ proteins. Surprisingly, we found that most TCRα proteins synthesized in $CD4^+CD8^+$ thymocytes during a 30 min pulse period did not survive 30 min of chase (Figure 5, bottom panels). In contrast, most TCRα proteins radiolabeled during the 30 min pulse period in splenic T cells were present following 30 min of chase (Figure 5, bottom panels). Thus, the survival of nascent TCRα proteins is markedly different in immature $CD4^+CD8^+$ thymocytes and splenic T cells, with most newly synthesized TCRα chains in $CD4^+CD8^+$ thymocytes undergoing rapid degradation shortly after their synthesis.

To determine if both assembled and unassembled (free) forms of TCRα proteins were degraded in $CD4^+CD8^+$ thymocytes, we performed sequential immunoprecipitations with anti-TCRα mAb to isolate TCRα chains associated with TCRβ proteins (Figure 5, top panels), followed by precipitation with anti-TCRβ mAb to capture free TCRα chains not assembled with TCRβ polypeptides (Figure 5, middle panels). As demonstrated, there was a marked difference in the ability of TCRα and β proteins to associate in $CD4^+CD8^+$ thymocytes versus splenic T cells (Figure 5, top panels). When normalized to equivalent amounts of nascent TCRβ chains, the number of TCRα chains found to be assembled with TCRβ proteins in $CD4^+CD8^+$ thymocytes was only one seventh to one eighth of that in splenic T cells. TCRα molecules that associated with TCRβ in either cell type became increasingly acidic and of higher molecular weight during 30 min of chase, consistent with processing of N-linked oligosaccharide side chains by Golgi glycosidases and glycosyltransferases (Figure 5, top panels). Free TCRα proteins that were not co-precipitated with TCRβ chains did not show evidence of Golgi processing and rapidly disappeared during 30 min of chase in lysates of $CD4^+CD8^+$ thymocytes and splenic T cells (Figure 5, middle panels).

In splenic T cells, most of the material that disappeared from the free TCRα pulse fraction (Figure 5, middle panels) could be accounted for in the TCRβ-associated chase fraction (Figure 5, top panels), indicating ongoing assembly during the chase period. However, not all TCRα chains were assembled during 30 min of chase in splenic T cells, as surviving free TCRα chains were clearly visible in chase groups of splenic T cell lysates (Figure 5, middle

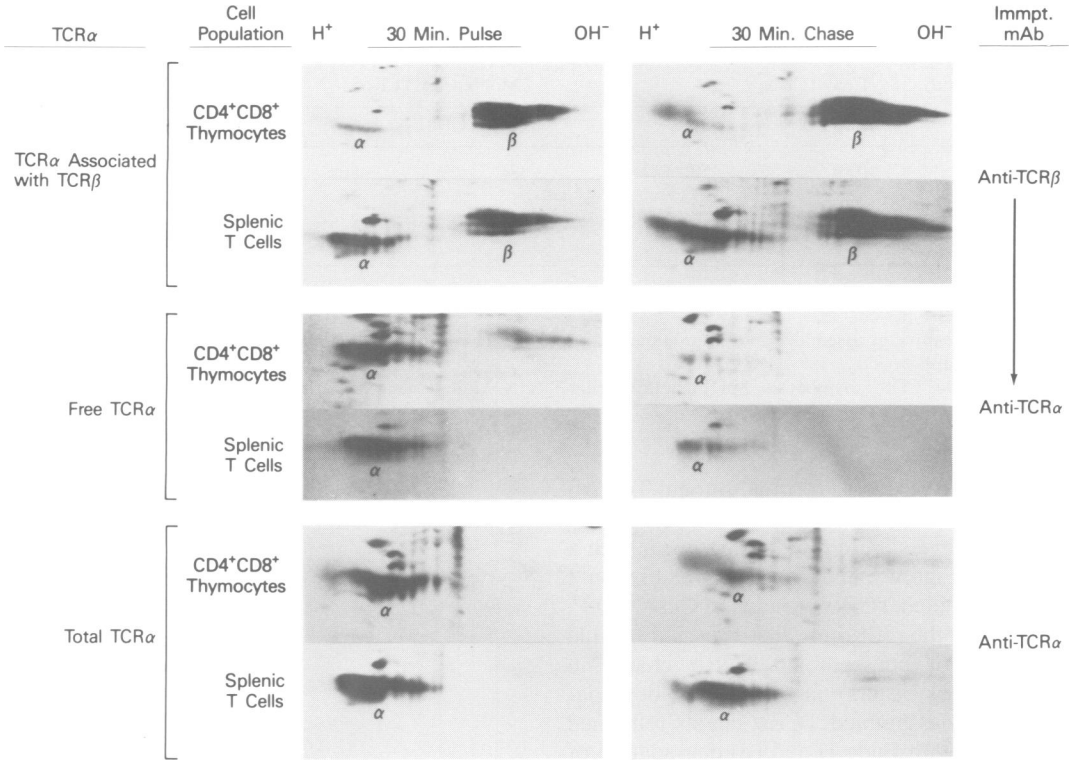

Fig. 5. Inefficient association of TCRα and β proteins in $CD4^+CD8^+$ thymocytes. Radiolabeled NP-40 lysates of $CD4^+CD8^+$ thymocytes and splenic T cells were sequentially immunoprecipitated with mAb to TCRβ to isolate TCRα proteins associated with TCRβ (**top panel**), followed by mAb to TCRα to isolate unassembled TCRα proteins (**middle panel**). In addition, lysates were precipitated with anti-TCRα mAb to isolate total TCRα proteins (**bottom panel**). Immunoprecipitated material was resolved by two-dimensional NEPHGE/SDS−PAGE under reducing conditions. Autoradiographs were exposed to show approximately equal amounts of protein precipitated by specific TCR mAb in $CD4^+CD8^+$ thymocyte and splenic T cell lysates. In the top panel, autoradiographs were exposed such that approximately equivalent amounts of TCRβ proteins were present, to assess TCRβ-associated TCRα chains. In the middle and bottom panels, autoradiographs were exposed such that approximately equivalent amounts of TCRα proteins were present in pulse-labeled material of $CD4^+CD8^+$ and splenic T cell lysates, to examine the fate of unassembled (middle panel) and total (top panel) TCRα chains. The positions of TCRα and β proteins are indicated.

panels). In contrast, in immature $CD4^+CD8^+$ thymocytes only trace amounts of material remained in the free TCRα chase groups (Figure 5, middle panels), without a concomitant increase in TCRβ-associated TCRα chains observed in $CD4^+CD8^+$ thymocytes during the chase period (Figure 5, top panels), indicating that unassembled TCRα chains were rapidly degraded in $CD4^+CD8^+$ thymocytes. Thus, these experiments demonstrate that association of TCRα and β proteins is quantitatively limited in $CD4^+CD8^+$ thymocytes relative to splenic T cells, with unassembled TCRα proteins being rapidly degraded in $CD4^+CD8^+$ thymocytes. However, TCRα chains that do assemble with TCRβ proteins in $CD4^+CD8^+$ thymocytes are stable and reach the Golgi apparatus, reflecting their assembly into TCR complexes that are competent to exit the ER.

Differential stability of nascent TCRα chains in $CD4^+CD8^+$ thymocytes and splenic T cells

To examine further the differential stability of nascent TCRα proteins in $CD4^+CD8^+$ thymocytes and splenic T cells, anti-TCRα immunoprecipitates of metabolically labeled NP-40 lysates from $CD4^+CD8^+$ thymocytes and splenic T cells were also examined by two-dimensional non-reducing/reducing SDS−PAGE in two separate experiments (Figure 6 and Table I). At the conclusion of the 30 min pulse period, nascent TCRα proteins existed

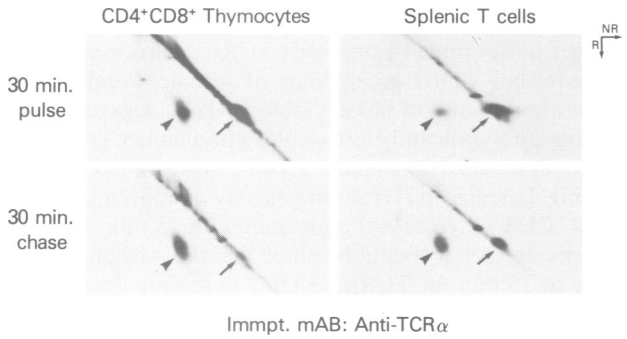

Fig. 6. Differential stability of nascent TCRα chains in immature $CD4^+CD8^+$ thymocytes and splenic T cells. Purified $CD4^+CD8^+$ thymocytes and splenic T cells were labeled in methionine-free medium containing [^{35}S]methionine at 1 mCi/ml for 30 min at 37°C and chased in complete medium containing excess cold methionine for an additional 30 min. Cells were solubilized in 1% NP-40, the lysates immunoprecipitated with mAb to TCRα and the precipitates analyzed by two-dimensional non-reducing/reducing SDS−PAGE. The positions of disulfide-linked TCRαβ heterodimers (arrowhead) and monomeric non-disulfide-linked TCRα proteins (arrow) are indicated.

in two forms in $CD4^+CD8^+$ thymocytes: as TCRαβ heterodimers (Figure 6, arrowhead) and as TCRα monomers (Figure 6, arrow). During the 30 min chase period, nearly all monomeric TCRα proteins disappeared, with few monomeric TCRα proteins associating with TCRβ

Table I. Differential stability of nascent TCRα proteins in immature $CD4^+CD8^+$ thymocytes and mature splenic T cells

Pulse (min)	Chase (min)	Total TCRα		Monomeric TCRα[a]		Dimeric TCRα[b]	
		$CD4^+CD8^+$[c]	SplT	$CD4^+CD8^+$	SplT	$CD4^+CD8^+$	SplT
30	0	100	100	84	86	16	14
	30	22	75	5	46	17	29
	60		68		22		46
	90		38		10		28

[a]Relative amounts of TCRα proteins existing as monomers is expressed as a percentage of total radiolabeled TCRα proteins detected at the conclusion of the 30 min pulse period.
[b]Relative amounts of TCRα proteins assembled into heterodimers is expressed as a percentage of total radiolabeled TCRα proteins detected at the conclusion of the 30 min pulse period.
[c]$CD4^+CD8^+$, immature $CD4^+CD8^+$ thymocytes; SplT, mature splenic T cells. Multiple autoradiographs of different exposures were scanned to ensure linearity.

chains during the chase period to form additional TCRαβ heterodimers. Thus, most unassembled TCRα monomers in $CD4^+CD8^+$ thymocytes were rapidly degraded, with a half-life of ~15 min (Figure 6 and Table I). Notably, only 22% of nascent radiolabled TCRα proteins in $CD4^+CD8^+$ thymocytes survived the 30 min chase period (Table I). The degradation of unassembled TCRα chains in $CD4^+CD8^+$ thymocytes occurs via a pre-Golgi degradation pathway as: (i) unassembled TCRα chains remained sensitive to digestion with Endo H and (ii) degradation of TCRα proteins was unaffected by NH_4Cl (data not shown).

In contrast to immature $CD4^+CD8^+$ thymocytes, 75–80% of nascent radiolabeled TCRα proteins in mature splenic T cells survived 30 min of chase. As demonstrated, most nascent TCRα proteins that existed as monomers after the pulse period in splenic T cells assembled with TCRβ chains to form TCRαβ heterodimers during the 30 min chase period and were not degraded (Figure 6 and Table I). Indeed, unassembled monomeric TCRα chains were relatively stable in splenic T cells, as TCRα monomers were still clearly visible following 30 min of chase. In fact, unassembled monomeric TCRα chains were detectable in anti-TCRα precipitates of splenic T cell lysates even after 90 min of chase (Table I). Thus, nascent TCRα chains are significantly less stable in immature $CD4^+CD8^+$ thymocytes than in mature T cells, with TCRα proteins having a median survival time of only 15 min in immature $CD4^+CD8^+$ thymocytes compared with 75 min in mature T cells. In fact, it should be noted that the median survival time of 75 min for TCRα proteins in splenic T cells is an underestimate of the survival of nascent TCRα proteins in the ER of mature T cells, as post-Golgi degradation of αβγδε TCR intermediates in lysosomes of splenic T cells accounts for the late loss of TCRα proteins which is seen as a decrease in dimeric TCRα proteins after 60 min of chase (Table I).

Differential survival of nascent TCRα and TCRβ proteins in $CD4^+CD8^+$ thymocytes

To determine if the instability of nascent TCRα chains in immature $CD4^+CD8^+$ thymocytes was unique to TCRα proteins, we next examined the stability of unassembled nascent TCRβ proteins. Cells were metabolically labeled with [^{35}S]methionine, solubilized in 1% NP-40 and the lysates sequentially precipitated with anti-TCRα mAb (to remove TCRαβ dimers), followed by precipitation with anti-TCRβ mAb. Immunoprecipitates were analyzed by two-dimensional non-reducing/reducing SDS−PAGE. As previously demonstrated, newly synthesized TCRα chains existed as TCRα heterodimers (Figure 7, thick arrow) and free monomeric chains (Figure 7, thin arrow) in $CD4^+CD8^+$ thymocytes, with free TCRα chains having a half-life of only 15 min. In contrast, most nascent free TCRβ chains radiolabled during the pulse period were still present following 90 min of chase in $CD4^+CD8^+$ thymocyte lysates, indicating that, unlike nascent TCRα proteins, nascent TCRβ proteins were quite stable within the ER of $CD4^+CD8^+$ thymocytes (Figure 7). Furthermore, it should be appreciated that the vast majority of TCRβ proteins were not disulfide-linked to any other protein in $CD4^+CD8^+$ thymocytes, as they existed as TCRβ monomers throughout the experimental period (Figure 7, thin arrow). Similar results were obtained for nascent TCRβ proteins in splenic T cells (data not shown).

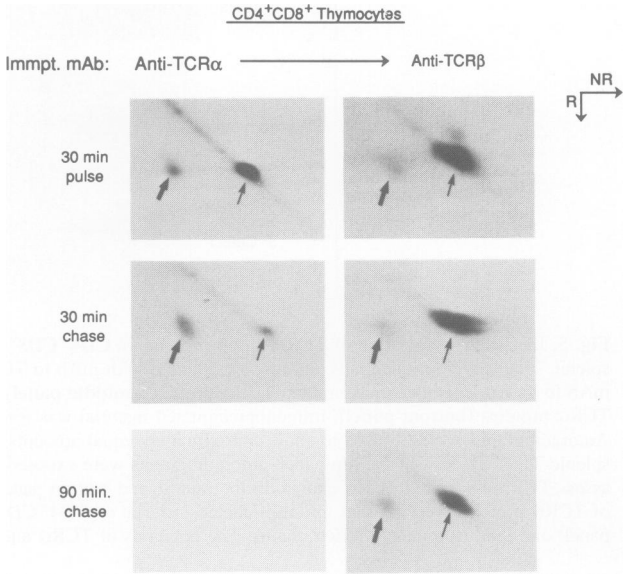

Fig. 7. Differential survival of nascent TCRα and β proteins in immature $CD4^+CD8^+$ thymocytes. Purified $CD4^+CD8^+$ thymocytes were metabolically labeled for 30 min and chased for the time period indicated, solubized in 1% NP-40 and the lysates immunoprecipitated with mAb to TCRα. Material not precipitated by two rounds of anti-TCRα mAb was then sequentially precipitated with anti-TCRβ mAb. Precipitates were analyzed by two-dimensional non-reducing/reducing SDS−PAGE. The positions of monomeric (arrow) and disulfide-linked (arrowhead) proteins are indicated.

The relative stability of nascent TCRβ proteins in

$CD4^+CD8^+$ thymocytes is consistent with the existence of a sizeable intracellular pool of unassembled TCRβ chains. Indeed, the existence of a pool of unassembled TCRβ proteins explains our observation (Figure 5, bottom panels) that anti-TCRα immunoprecipitates from both thymus and spleen contained labeled TCRα proteins, but did not contain labeled TCRβ proteins. Thus, formation of TCRαβ heterodimers in both $CD4^+CD8^+$ thymocytes and splenic T cells involves the pairing of nascent TCRα proteins with a pre-existent pool of TCRβ chains.

Differential survival of nascent transgenic TCRα proteins in $CD4^+CD8^+$ thymocytes and splenic T cells

Since immature $CD4^+CD8^+$ thymocytes represent pre-selection thymocytes, inefficient association of TCRα and TCRβ proteins might have reflected a high frequency of 'mismatched' TCRα and β combinations that were unable to pair efficiently. Consequently, we compared the association of a matched set of Vα11 and Vβ3 transgenic proteins in $CD4^+CD8^+$ thymocytes and splenic T cells from TCR transgenic mice (Kaye *et al.*, 1989). Immature $CD4^+CD8^+$ thymocytes and splenic T cells from 'AND' TCR transgenic mice were metabolically labeled for 30 min, chased for an additional 30 min, solublized in 1% NP-40 and the lysates precipitated with mAb to TCRVα11. As is evident, the stability of TCRVα11 polypeptides is markedly different in immature $CD4^+CD8^+$ thymocytes relative to mature T cells. Similar to our findings with endogenous TCRα proteins in non-transgenic mice, most newly synthesized TCRVα11 chains survived 30 min of chase in splenic T cells; in contrast, the majority of TCRVα11 proteins synthesized in $CD4^+CD8^+$ thymocytes during the pulse period did not survive 30 min of chase, indicating that most had failed to assemble with TCRβ and were degraded (Figure 8A). That this was indeed the case is seen in Figure 8B. Fewer than 30% of nascent TCRVα11 transgenic proteins in $CD4^+CD8^+$ thymocytes associated with TCRVβ3 transgenic proteins during the pulse period, as 70% of labelled TCRVα11 proteins failed to be immunoprecipitated by mAb to TCRβ and were captured only after subsequent immunoprecipitation with mAb to TCRVα11 (Figure 8B, lane 3). The unassembled TCRVα11 transgenic proteins were rapidly degraded in $CD4^+CD8^+$ thymocytes and failed to survive 30 min of chase (Figure 8B, lane 6). Thus, even matched pairs of TCRα and β proteins fail to efficiently associate in $CD4^+CD8^+$ thymocytes. Moreover, these studies show that the stability of a single transgenic TCRα protein is markedly different in immature $CD4^+CD8^+$ thymocytes versus mature splenic T cells, indicating that post-translational mechanisms regulate the survival of nascent TCRα chains in developing thymocytes.

Retention within the ER is not sufficient to cause rapid degradation of nascent TCRα chains

Finally, we wished to determine if unassembled TCRα chains were rapidly degraded in $CD4^+CD8^+$ thymocytes simply because they were incompetent to exit the ER. For these studies we used the BW5147 thymoma cell line, because nascent TCRα proteins are retained within their ER as unassembled proteins (Lippincott-Schwartz *et al.*, 1989b). BW cells were metabolically labeled, solubilized

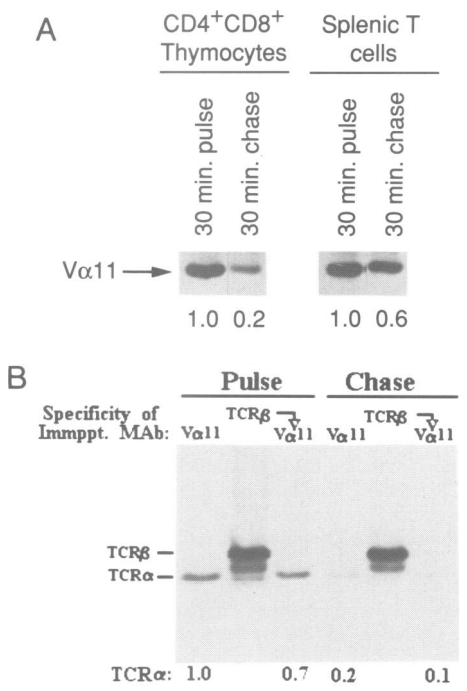

Fig. 8. Inefficient association of paired TCRα and β transgenic proteins in $CD4^+CD8^+$ thymocytes. (**A**) Purified $CD4^+CD8^+$ thymocytes and splenic T cells from AND TCR transgenic mice were labeled in methionine-free medium containing [^{35}S]methionine at 1 mCi/ml for 30 min at 37°C and chased in complete medium containing excess cold methionine for an additional 30 min. Cells were solublized in 1% NP-40 and the lysates were precipitated with mAb to TCRVα11 pre-adsorbed on protein G beads to isolate total TCRVα11 proteins. Precipitates were analyzed on one-dimensional SDS−PAGE under reducing conditions. The relative amounts of TCRVα11 molecules were determined by densitometric scanning, with the total amount of TCRVα11 metabolically labeled during the pulse period set at 1.0. Multiple exposures of autoradiographs were scanned to ensure linearity. (**B**) Purified $CD4^+CD8^+$ thymocytes from AND TCR transgenic mice were metabolically labeled as described in (A). NP-40 lysates were precipitated with mAb to TCRVα11 to isolate total TCRVα11 proteins. Parallel lysates were sequentially immunoprecipitated with mAb to TCRβ to isolate TCRVα11 proteins assembled with TCRβ, followed by mAb to TCRVα11 to purify unassembled TCRVα11 proteins. The relative amounts of TCRVα11 molecules were determined by densitometric scanning, with the total amount of TCRVα11 metabolically labeled during the pulse period set at 1.0. Multiple exposures of autoradiographs were scanned to ensure linearity.

in 1% NP-40 and the lysates precipitated with anti-TCRα mAb. Immunoprecipitated samples were digested with Endo H and analyzed on SDS−polyacrylamide gels under reducing conditions. As demonstrated in Figure 9, TCRα proteins synthesized in BW cells remained sensitive to digestion with Endo H following 30 min of chase, consistent with their localization within the ER (Lippincott-Schwartz *et al.*, 1989b). However, even though these TCRα chains were retained within the ER, they were not degraded, demonstrating that retention within the ER is not sufficient for rapid degradation of nascent TCRα chains.

We have recently found that inhibition of glucose removal from core oligosaccharide side chains prevents association of nascent TCRα proteins with the resident ER protein calnexin, resulting in malfolded TCRα polypeptide chains (Kearse *et al.*, 1994). To demonstrate that BW cells are capable of degrading nascent TCRα chains, we cultured BW cells with the glucosidase inhibitor castanospermine

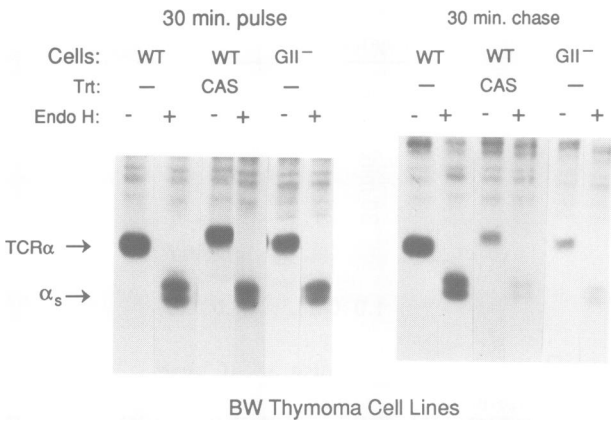

Fig. 9. Retention within the ER is not sufficient for rapid degradation of nascent TCRα chains. Wild-type BW thymoma cells were cultured in medium or castanospermine for 2 h at 37°C, metabolically labeled with [^{35}S]methionine for 30 min and chased for the time period indicated. Castanospermine was used at a concentration of 100 μg/ml. Glucosidase II-deficient mutant BW cells were metabolically labeled as above except that no pretreatment was performed. Cells were solubilized in 1% NP-40 and the lysates precipitated with anti-TCRα mAb. Immunoprecipitates were either mock treated or digested with Endo H and analyzed under reducing conditions. The positions of TCRα chains are indicated. α_S, Endo H-sensitive TCRα proteins.

(cas) (Kaushal and Elbein, 1994), to interfere with oligosaccharide processing and proper protein folding. Indicative of the action of cas, TCRα chains synthesized in cas-treated BW cells migrated more slowly on SDS–polyacrylamide gels compared with TCRα chains made in untreated BW cells, because of the persistence of glucose residues on core oligosaccharides (Figure 9). Most importantly, TCRα chains synthesized in cas-treated BW cells were remarkably unstable relative to TCRα chains synthesized in untreated BW cells, with only trace amounts of radiolabled TCRα proteins remaining following 30 min of chase. In addition, we examined the stability of nascent TCRα chains synthesized in a mutant BW cell line PHAR2.7 that is deficient in glucosidase II enzyme activity (Reitman *et al.*, 1982). Similar to what was observed in cas-treated BW wild-type cells, nascent TCRα chains were rapidly degraded in PHAR2.7 cells, having a half-life of approximately 15 min. Thus, these experiments show that retention within the ER is not sufficient to cause degradation of nascent TCRα proteins by ER proteolytic enzymes. However, as demonstrated, perturbation of quality control mechanisms that operate within the ER to ensure proper folding of nascent glycoproteins results in markedly rapid degradation of TCRα proteins.

Discussion

The present study describes a novel mechanism for quantitatively regulating the expression of a multisubunit receptor complex during development. We found that low TCR expression in immature $CD4^+CD8^+$ thymocytes is due to inefficient assembly of complete TCR complexes, which, in turn, results from impaired association of TCRα with TCRβ proteins. Inefficient formation of TCRαβ heterodimers in $CD4^+CD8^+$ thymocytes results from the uniquely rapid degradation of nascent TCRα proteins within the ER of immature thymocytes. Thus, low surface TCR expression in immature $CD4^+CD8^+$ thymocytes results from instability of nascent TCRα proteins within the ER, which severely limits the formation of complete TCR complexes in these cells.

The fate of newly assembled TCR complexes in immature $CD4^+CD8^+$ thymocytes has been uncertain (Bonifacino *et al.*, 1990a; Kosugi *et al.*, 1992). The present results demonstrate that complete TCR complexes exit the ER of immature $CD4^+CD8^+$ thymocytes, transit to at least the *trans*-Golgi compartment and are relatively stable, consistent with their being expressed on the cell surface. While addition of TCRζ chains is the final step in TCR assembly, the high rate at which TCRζ chains are degraded in immature $CD4^+CD8^+$ thymocytes was found to reflect the low rate at which they are assembled into complete TCR complexes and not to reflect instability of TCRζ-containing TCR complexes in $CD4^+CD8^+$ thymocytes, as has been suggested (Kosugi *et al.*, 1992). Similarly, the high rate at which TCR components are degraded in the ER of immature $CD4^+CD8^+$ thymocytes reflects the low rate at which they are assembled into incomplete αβγδε intermediates and complete αβγδεζ complexes and does not reflect the retention of complete TCR complexes within the ER of immature $CD4^+CD8^+$ thymocytes, as we previously suggested (Bonifacino *et al.*, 1990a).

Inefficient association of TCRα and β proteins and the markedly rapid degradation of unassembled TCRα chains is a novel assembly defect unique to immature $CD4^+CD8^+$ thymocytes, having not been observed in any T cell line in which TCR assembly has been studied. Indeed, the degradation of unassembled TCRα chains in $CD4^+CD8^+$ thymocytes is markedly accelerated relative to that previously observed in either T cell lines or non-T cell lines expressing a transfected TCRα gene product (Minami *et al.*, 1987; Bonifacino *et al.*, 1988; Chen *et al.*, 1988; Klausner *et al.*, 1990). The assembly defect in immature $CD4^+CD8^+$ thymocytes is independent of the clonotypic specificities expressed by TCRα and β proteins, as even a matched pair of TCRα and TCRβ transgenic proteins failed to associate efficiently. Rather, the inefficiency of TCRα and β association reflects assembly constraints on TCR proteins present within the ER of immature $CD4^+CD8^+$ thymocytes.

It has recently been reported that the rate of synthesis of TCRα chains is lower in immature $CD4^+CD8^+$ thymocytes than in mature thymocytes (Kosugi *et al.*, 1992). Because the present results demonstrate that nascent TCRα proteins are unstable in immature $CD4^+CD8^+$ thymocytes, most of the nascent TCRα proteins that were synthesized during the metabolic labeling period would have been degraded, which would have caused Kosugi and co-workers to significantly underestimate the rate of TCRα synthesis in immature $CD4^+CD8^+$ thymocytes. It is precisely because nascent TCRα proteins are more rapidly degraded in immature than mature T cells that the present study has not attempted to compare TCRα synthetic rates directly between immature and mature T cells. However, we think that the rate of TCRα protein synthesis is probably comparable in immature and mature T cells, because these cells have been found to have equivalent amounts of TCRα mRNA (Maguire *et al.*, 1990).

Instability of unassembled TCRα proteins in $CD4^+CD8^+$ thymocytes may relate to the recent sugges-

tion that TCRα proteins are inefficiently inserted into the ER lipid bilayer because their transmembrane region is thermodynamically unstable as an α-helix (Shin *et al.*, 1993). Because an ER degradation sequence of TCRα proteins is localized in the transmembrane domain (Bonifacino *et al.*, 1990b,c), failure to insert into the ER lipid bilayer might expose this region to degradative enzymes within the ER lumen. Thus, interaction of TCRα proteins with other ER proteins that are necessary for proper insertion of TCRα chains into the lipid bilayer may be impaired in $CD4^+CD8^+$ thymocytes relative to mature T cells. Indeed, we observed in the present study that interference with oligosaccharide processing, which interferes with ER control mechanisms that ensure proper folding of nascent glycoproteins, results in the rapid degradation of nascent TCRα proteins within the ER of BW thymoma cells. We have previously observed that removal of glucose residues on core oligosaccharides is necessary for association of nascent TCR glycoproteins with the resident ER protein calnexin (Kearse *et al.*, 1994), an association which is thought to be necessary for proper protein folding, but which might also function to anchor nascent TCRα proteins in the ER lipid bilayer. Thus, it is possible that potential deficiencies in immature $CD4^+CD8^+$ thymocytes in the ability to remove glucose residues from oligosaccharide side chains of nascent TCRα proteins might be the basis for the instability of nascent TCRα proteins in the ER of immature $CD4^+CD8^+$ thymocytes. Alternatively, other as yet undefined lesions may exist in the ER quality control system of $CD4^+CD8^+$ thymocytes that interfere with proper folding of nascent TCRα proteins, resulting in their rapid degradation.

The existence of a disulfide-linked heterodimer on early thymocytes consisting of the TCRβ chain and a novel 33 kDa glycoprotein (gp33) has recently been reported (Groettrup *et al.*, 1993). We would like to emphasize that the failure of TCRα proteins to assemble with TCRβ proteins in immature $CD4^+CD8^+$ thymocytes, as observed in the present study, cannot be due to competition for dimerization with TCRβ among TCRα, gp33 and, perhaps, other proteins, because the vast majority of nascent TCRβ proteins in $CD4^+CD8^+$ thymocytes remained monomeric and unassembled. Moreover, the amount of gp33 present in early thymocytes of normal mice was below detectable levels and was only found in overexpressing pre-T cell lines that failed to express TCRα (Groettrup *et al.*, 1993).

Finally, it is interesting to note that the differential stabilities of nascent TCRα and TCRβ proteins in immature thymocytes parallels the efficacy with which their gene loci are allelically excluded, a parallel pointed out to us by Dr Patrice Marche (Pasteur Institute, Paris, France). In contrast to the TCRβ gene locus, which is effectively allelically excluded, the TCRα gene locus is inefficiently excluded, with many T cells expressing two different TCRα proteins (Padovan *et al.*, 1993). Thus, it is interesting to speculate that inefficient allelic exclusion of TCRα genetic loci in immature thymocyes may result from instability of newly synthesized TCRα proteins within the ER, limiting the number of TCRα proteins available for feedback regulation and allelic exclusion of the TCRα gene locus.

In conclusion, the present study demonstrates that TCR assembly is dynamically regulated during intrathymic differentiation. The post-translational regulation of TCR assembly in $CD4^+CD8^+$ thymocytes defines a novel mechanism for quantitatively regulating expression of a multisubunit complex during development. Whether or not expression of other multisubunit receptor complexes are developmentally regulated via a similar mechanism remains to be elucidated.

Materials and methods

Animals, cell preparation and reagents

$CD4^+CD8^+$ thymocytes were isolated from C57BL/6 mice by adherence to plastic plates coated with anti-CD8 mAb (83-12-5), and were typically >96% $CD4^+CD8^+$, as described (Nakayama *et al.*, 1990). Purified splenic T cells were obtained by incubating single cell suspensions of spleen cells on rabbit anti-mouse Ig (Organon Technika-Cappel, Malvern, PA)-coated tissue culture plates for 60 min at 37°C, followed by isolation of non-adherent cells. The resultant cell populations were typically >95% $CD3\varepsilon^+$ as determined by surface staining with mAb to CD3ε. Transgenic 'AND' TCR mice (Kaye *et al.*, 1989) were kindly provided by Dr Stephen Hedrick. BW thymoma cell lines (Hyman and Stallings, 1974) and glucosidase II-deficient PHAR2.7 cell lines (Reitman *et al.*, 1982) were maintained by weekly passage in RPMI 1640 medium containing 10% fetal calf serum as originally described. Castanospermine was purchased from Calbiochem (La Jolla, CA) and was used at a final concentration of 100 μg/ml.

Antibodies

The following mAbs were used in this study: anti-CD3ε 145-2C11 (Leo *et al.*, 1987), anti-TCRα H28-710 (Kubo *et al.*, 1989), anti-TCRβ H57-597 (Kubo *et al.*, 1987), anti-TCRζ H146.968 (Punt *et al.*, 1991), anti-TCRVα11 RR8-1 (Pharmingen) and anti-2B4-TCRα A2B4 (Samelson *et al.*, 1983). The following antisera were used: anti-CD3δ R9 (Samelson *et al.*, 1986) and anti-ζ 551 (Cenciarelli *et al.*, 1992).

Metabolic labeling

For metabolic labeling, cells were resuspended at $5–10\times10^6$ cells/ml in methionine-free RPMI 1640 media (Biofluids, Rockville, MD) containing 10% fetal calf serum and 10 mCi [^{35}S]methionine (Trans ^{35}S Label; Irvine, CA) for 30 min at 37°C. For chase experiments, cells were washed twice in chase medium (RPMI medium containing 10% FCS and excess cold methionine at 150 μg/ml), resuspended at their original concentration in fresh chase media and placed at 37°C for the time period indicated. For experiments using glucosidase inhibitors, cells were pre-incubated with media or castanospermine (Calbiochem, La Jolla, CA) (final concentration 100 μg/ml) for 2 h prior to metabolic labeling. The presence of the inhibitor was maintained throughout the pulse and chase periods.

Lectin affinity chromotography

Cells were lysed in 1% digitonin and diluted with buffer to yield a final detergent concentration of 0.5%. Half of the lysate was added to *Ricinus communis* agglutinin (RCA)-conjugated beads (EY Laboratories) which had been washed three times with PBS and once in 0.5% digitonin lysis buffer and incubated for 16 h at 4°C. Samples were centrifuged and the supernatants removed and placed on ice. RCA-bound material was eluted by the addition of 200 mM β-lactose (Sigma) in digitonin wash buffer for 3–4 h at 4°C. Samples were centrifuged, the supernatants removed and binding proteins were immunoprecipitated.

Immunoprecipitation, gel electrophoresis and immunoblotting

Cells were solubilized in lysis buffer (20 mM Tris, 300 mM NaCl, 10 mM iodoacetamide, 20 μg/ml leupeptin, 40 μg/ml aprotinin) containing 1% digitonin (Wako) or 1% NP-40 (Calbiochem) for 25 min at 4°C. Cell lysates were clarified by centrifugation to remove insoluble material. For immunoprecipitation, lysates were mixed with appropriate antibodies pre-adsorbed to protein A–Sepharose or protein G–Sepharose beads (Pharmacia) and incubated for at least 3 h at 4°C. Sequential immunoprecipitations and Endo H digestion were performed as previously described (Kearse *et al.*, 1993). For immunoblotting, material was transferred to nitrocellulose and blots probed with a 1:200 dilution of antisera in PBS containing 5% milk and 0.02% NaN_3, followed by ^{125}I-labelled protein A (1 mCi/ml) (ICN, Irvine, CA). Samples were analyzed by two-

dimensional non-reducing/reducing SDS–PAGE and two-dimensional NEPHGE/SDS–PAGE as previously described (Bonifacino *et al.*, 1990a).

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