Domains of the TCRβ-Chain Required for Early Thymocyte Development

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Summary

The T cell receptor β (TCRβ) chain controls the developmental transition from CD4-CD8- to CD4+CD8+ thymocytes. We show that the extracellular constant region and the transmembrane region, but not the variable domain or cytoplasmic tail of the TCRβ chain are required for this differentiation step. TCRβ mutant chains lacking the cytoplasmic tail can be found at the cell surface both in functional TCR/CD3 complexes and in a GPI-anchored monomeric form indicating that the cytoplasmic tail of the TCRβ chain functions as an ER retention signal. The concordance between cell surface expression of the mutant chains as TCR/CD3 complexes and their capacity to mediate thymocyte differentiation supports the CD3 mediated feedback model in which preTCR/CD3 complexes control the developmental transition from CD4-CD8- to CD4+CD8+ thymocytes.

The α and β chains of the TCR play a central role in the development of αβ T cells (1-3). Both chains are members of the Ig superfamily and are encoded by gene segments that undergo rearrangements during T cell differentiation to yield functional genes (4). Rearrangement of both, TCR and Ig genes, requires the expression of the recombination activating genes RAG-1 and RAG-2 (5, 6).

During T cell development, rearrangement at the TCRβ locus precedes rearrangement at the TCRα locus (7, 8). In transgenic mice, expression of a functional TCRβ transgene was shown to block rearrangement and expression of endogenous TCRβ genes, indicating that TCRβ genes are subject to allelic exclusion (9). Expression of the TCRβ protein was shown to be required for mediating allelic exclusion at the TCRβ locus (10). However, a mutant TCRβ chain lacking the variable domain (ΔV-TCRβ), efficiently blocks rearrangements of endogenous TCRβ alleles, while leaving recombination of the TCRα genes unimpaired (10, 11).

How does a TCRβ chain block V-DJ rearrangements? Studies in TCRβ transgenic mice are most consistent with the feedback model, first proposed for allelic exclusion at the Ig heavy chain locus (12). Before rearrangement at Ig light chain loci, Ig heavy chains assemble with surrogate light chains (VpreB and λ5) as well as mb-1/B29 heterodimers (13-16). This pre-B cell receptor complex appears at very low levels at the cell surface and is thought to induce the development from pro-B cells to pre-B cells, concomitant with the shut off of the Ig heavy chain loci through specific target elements (17-20). The identification of a surface-expressed pre-T cell receptor complex analogous to the pre-B cell receptor complex, has allowed extension of this model to the T cell lineage (21-24). Early in pre-T cell development, the pre-TCR complex is thought to signal a functional V-DJ rearrangement, impairing further V-DJ rearrangements. As is clear from studies of recombination-deficient mice reconstituted with a functional TCRβ transgene (3, 22, 25) or with a mutant ΔV-TCRβ transgene (26), the pre-TCR complex appears to be involved in further differentiation of pre-T cells including downregulation of the interleukin 2 receptor α (IL2-Rα) chain, upregulation of CD4 and CD8 coreceptors, enhanced transcription at the TCRα locus and expansion of the thymocytes to normal numbers. More recently, a novel 33-kD glycoprotein (pTot) has been identified as the binding partner of the TCRβ glycoprotein. This heterodimer is found at very low levels at the cell surface of pre-T cells (27). Subsequent cloning and gene-targeting of the pTot gene in mice demonstrated the crucial role of preTot chain in pre-T cell development (28, 29).

Evidence for an important role of CD3 components in signal transduction by the pre-TCR/CD3 complex has been provided (26, 30, 31). CD3 components are present at the surface of developmentally arrested thymocytes of RAG-deficient mice in the absence of TCR chains. Cross-linking of the CD3 modules on differentiation arrested CD4+CD8+ thymocytes of RAG-1-/- mice in vitro (30) or in vivo (26, 31) with anti-CD3ε mAb induces the tran-
siton from CD4<sup>+</sup>CD8<sup>+</sup> thymocytes to CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, concomitant with all the changes described for recombination-deficient mice reconstituted with a functional TCR<sub>B</sub> transgene or the mutant ΔV-TRC<sub>B</sub> transgene (see above). Thus, cross-linking of the CD3 modules on CD4<sup>+</sup>CD8<sup>+</sup> thymocytes apparently mimics the signaling mediated by the pre-TCR complex.

Earlier experiments have shown that the variable domain of the TCR<sub>B</sub> chain is not required to induce early thymocyte development (10, 11, 26). To determine the domains required to induce pre-T cell development, we have generated five different mutant TCR<sub>B</sub> transgenic mice with defined alteration in the TCR<sub>B</sub> constant region.

Materials and Methods

Construction of Mutant TCR<sub>B</sub> Transgenes

Starting material for the construction of the ββββΔ, ββαα, βααβ, and αααα mutants (see Fig. 1) was a 20-kb Asp718 (KpnI) TCR<sub>B</sub> fragment from the cosmID clone cosHYB9-1.14-5 (9). Mutations were restricted to the 6.1-kb BamHI fragment containing all the four exons of the C<sub>B2</sub> constant region; transcriptional control elements such as the TCR<sub>B</sub> promoter and the 3′ TCR<sub>B</sub> enhancer region as well as the functional VDJ region were not altered.

Construction of a Mutant TCR<sub>B</sub> Transgene Lacking the Cytoplasmic Tail (ββββΔ). The ββββΔ protein was generated by converting the 2nd codon of the 4th TCR<sub>B</sub> constant exon into a TAG stop codon that deletes three lysines, an asparagine and a serine. After three sequential subcloning steps, first a 6.1-kb C<sub>B2</sub>, BamHI fragment, second a 1,940-kb PstI-HpaI fragment and third a 322-bp EcoRV fragment containing the TCR<sub>B</sub> constant exon 4 encoding the putative cytoplasmic tail of the TCR<sub>β</sub> chain was cloned into pSP72. By recombinant PCR the second codon AAG was mutated into a TAG stop codon, which introduces a BamHI fragment, and 322-bp PstI-HpaI fragment and third a 1.940-kb-PstI-HpaI fragment containing the signal mediated by the pre-TCR complex.

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DNA and RNA Blot Analysis

For Southern blot analysis 30 μg of total genomic DNA of each mouse was digested with restriction enzymes as recommended by the supplier, separated on 0.6% agarose gels, and transferred to nitrocellulose. Filters were hybridized to 32P-labeled probes and washed as described (36). Probes used for DNA analysis were Vβ8.1 (37) and Jβ2 (38). The final wash was at 0.1X SSC, 60°C and 0.1X SSC, 42°C, respectively.

For Northern blot analysis 20 μg of total RNA, prepared by the LiCl-urea method, was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes (39). Probes for Vβ8.1 (37), CX (40), CB (41), and actin (42) were 32P-labeled by random priming, hybridization conditions were as described (36) with the addition of 1% SDS to all solutions. The final wash was at 0.1X SSC, 60°C. Filters were exposed with Kodak X-Omat S or XAR-5 films at −70°C using intensifier screens.

Phospholipase C Treatment of Thymocytes

Thymocytes from ββββ, βββΔ, and βββ-P1 transgenic mice (106/ml) were incubated for 40 min at 37°C in complete Iscove’s medium either in the presence or absence of 1 U/ml phospholipase C (PLC) (isolated from Bacillus cereus).

Antibodies and Immunofluorescence

Anti-CD3ε 145-2C11 (43), anti-TCRβ mAb H57-597 (44), anti-Vβ6 mAb 44-22-1 (45), and anti-Vβ8 mAbs KJ16-133 (46) and F23.1 (47) were purified from culture supernatants by HPLC. Purified Ig and FITC- or biotin-conjugates thereof were used for immunoprecipitation and immunofluorescence analysis as indicated. The mAbs directed against CD4 (clone RM-4-5), CD8 (clone 53-6.7) and Vβ2 (clone B20.6) were purchased from Pharmingen. Flow cytometry was performed on a FACScan® (Becton Dickinson, Mountain View, CA), using lysls II software. For double labeling FITC- and PE or biotin-conjugated mAb were used. Cells incubated with biotin-conjugated mAb were subsequently stained by incubation with PE-conjugated streptavidin. Data are depicted as contour plots (log density 50%, threshold 1%, 1 X smoothed) or dot plots.

Radiolabeling, Immunoprecipitation, and Gel Electrophoresis

For metabolic labeling splenocytes of transgenic and non-transgenic FVB mice were cultured in 15 ml Iscove’s medium supplemented with 10% dialyzed serum and counted. Lymphocytes (20-30 × 106) were labeled metabolically for 4 hours with 100 μCi [35S]methionine in 1 ml methionine-free RPMI-medium in a humidified incubator at 37°C, 5% CO2. Subsequently the cells were harvested and lysed for 30 min at 4°C in NP-40 containing lysin buffer (50 mM triethanolamine-Cl, pH 7.5, 5 mM MgCl2, 1 mM PMSE, and 0.5% NP-40). Nuclear debris was removed by centrifugation for 15 min at 13,000 g.

Surface iodination, immunoprecipitation, SDS-PAGE, and autoradiography were carried out as described (11).

Results

The Experimental System. To dissect the domains within the TCRβ constant region which are required to induce pre T cell development, five different mutant TCRβ chain genes were constructed. The structure of each mutant TCR transgene product is illustrated in Fig. 1.

For transgenesis, the FVB mouse inbred strain was used. FVB mice lack Vβ8 segments as demonstrated by Southern blot and flowcytometric analysis, allowing specific detection of the Vβ8.2 domain common to all mutant TCR chains. To investigate whether the Vβ8.2 domain had any detrimental effects on T cell development in the FVB background, e.g., by Vβ8-specific superantigen(s), TCRβ transgenic C57BL/10A mice were crossed with FVB mice. The T cell compartments in TCRβ transgenic FVB × C57BL/10A F1 and C57BL/10A mice did not show any changes in the size of Vβ8.2+ T cell subsets in thymus, spleen and lymph nodes (data not shown).

Expression of the mutant transgenes was determined by Northern blot analysis using total RNA extracted from spleens of different founder lines and a Vβ8 specific probe. The 3' untranslated region of the TCRα mRNA is ~200 bp longer than that of the 3' untranslated region of the TCRβ message, whereas these two mRNAs are similar in length.

![Figure 1. Schematic illustration of the proteins encoded by the mutant TCR transgenes. TCRα protein (black, dashed) and TCRβ protein (ββββ, MAD) share a common basic 5-domain structure: V, variable domain; Ig, Ig-like domain; CP, connecting peptide, TM, transmembrane region; CT, cytoplasmic tail. Beside the non-mutant TCRβ transgene and the mutant ββββ chain, five additional alterations were introduced into the TCRβ constant regions. The ββββ mutant resembles a TCRβ protein lacking its cytoplasmic tail and the βββ-P1 mutant comprises the three extracellular constant regions of the TCRβ chain and the first 13 α of the TCRβ transmembrane region connected to the PI-anchor sequence of Q7. The βββααα, βααββ, and βααααα mutants were generated by domain swapping. The first β resembles the TCRβ variable domain, the second, third, fourth, and fifth letter indicates the origin of the extracellular constant Ig-like domain, the connecting peptide, the transmembrane region and the cytoplasmic tail, respectively. Δ indicates deletion of that particular region.](https://example.com/figure1.png)
in the other regions. As expected, ββααα and βαβββ transcripts co-migrated at the position of mature TCRα transcripts (1.5 kb) whereas the βββββ and βαβββ transcripts ran at the position of mature TCRβ transcripts (1.3 kb). The size of the βββββ mRNA was also in accordance with the structure of the βββββ transgene (not shown). Hybridization patterns with 32P-labeled probes specific for the TCRα and TCRβ constant regions were in accordance with the structure of the TCR α/β chimeric transgenes. The lack of detection of endogenous TCRβ transcripts is not due to allelic exclusion but to the relatively short exposure of the autoradiograph in view of the relatively high expression of the mutant TCRβ transgenes (Fig. 2 A).

Expression of the mutant TCR chains was further determined by SDS-PAGE analysis of Vβ8-specific immunoprecipitates from NP40-lysates of metabolically labeled Con A activated splenocytes. T cell enriched splenocytes from non-transgenic FVB mice and transgenic βββββ, ββααα, βαβββ, and βαααα mice were used. Immunoprecipitates were analyzed under reducing conditions on a 10-15% SDS-PAGE gradient gel. Whereas T cells from FVB mice lack V8-specific polypeptides, the T cells from the TCRβ mutant mice were found to express anti-Vβ8 reactive glycoproteins of the expected sizes (Fig. 2 B).

The ββααα Transgene Perturbs the Transition from the CD4+CD8- Stage to the CD4+CD8+ Stage. To study the effects of the different mutant TCR transgenes on thymocyte development, we first compared the number of thymocytes in the mutant transgenic lines. In control FVB mice, the number of thymocytes found in different mutant transgenic lines was comparable to that of non-transgenic mice (Fig. 3 A). In ββααα transgenic mice the number of thymocytes was reduced to 36 ± 8 % (n = 6) compared to non-transgenic control littermates (n = 7). To assess which developmental stages were affected by the expression of the ββααα chain, we determined the size of the different CD4+CD8 thymocyte compartments of control and mutant mice (Fig. 3, B and D). Although the absolute number of CD4+CD8- thymocytes in ββααα transgenic mice was normal, the number of the CD4+CD8+, CD4+CD8- and CD4-CD8+ subsets were clearly reduced, suggesting that the ββααα chain interferes either with the expansion of CD4+CD8+ thymocytes or with the transition of thymocytes from the CD4+CD8- to the CD4+CD8+ stage. Mature αβ T cells were significantly reduced in thymus, lymph nodes and spleen of ββααα transgenic mice (Fig. 3, C and D). In all other mutant transgenic lines analyzed, the different thymocyte compartments were comparable to those of non-transgenic FVB mice (Fig. 3, A–D).

Usage of Endogenous Vβ Gene Segments in Mutant TCR Transgenic Mice. Two color immunofluorescence analysis using anti-Vβ2 and anti-Vβ6 mAbs was performed to assess functional rearrangements of endogenous alleles. In non-transgenic FVB mice the αβ T cell subsets expressing Vβ2- and Vβ6-domains represent 13.8 ± 0.7 % and 9.8 ± 0.6 % (n = 28) of mature peripheral αβ T cells, respectively (Fig. 4).

However, in βββββ transgenic FVB mice the Vβ2+ and Vβ6+ subsets constituted only 0.2 ± 0.1% and 1.7 ± 0.8 % of mature αβ T cells, respectively (n = 12). These data strongly suggest that the βββββ mutant protein product efficiently prevents rearrangements of endogenous TCRβ alleles. Based on the usage of Vβ2 and Vβ6 gene segments in control FVB mice, the efficacy with which the βββββ transgene prevented surface expression of rearranged Vβ2- and Vβ6- gene segments was 99% and 83%, respectively.

In ββααα transgenic FVB mice, the Vβ2-subset com-
Figure 3. Quantitative comparison of the αβ T cell compartment in mutant TCR transgenic mice. (A) Relative number of thymocytes in mutant TCR transgenic mice. The number of thymocytes was determined by flow cytometry on a Sysmex F800 and compared to nontransgenic littermates and/or age-matched FVB mice. In βββαα transgenic mice, the number of thymocytes was found to be reduced to 36 ± 8% of control mice. The thymocyte cellularity in all other mutant transgenic mice, including the βββ-PI transgenics (not shown) does not differ from non-transgenic littersmates.

Number below each bar indicates the number of mice analyzed. (B) CD4/CD8 thymocyte subsets in mutant TCR transgenic mice. The percentage of CD4+CD8 double-negative (DN), CD4+CD8 double-positive (DP) and CD4+CD8 single-positive (SP) thymocyte subsets compared to the absolute number in FVB controls are depicted. The subsets were measured by two-color immunofluorescence and analyzed using Lysys II software (Becton Dickinson). Although the number of DN pro-thymocytes is normal in βββαα transgenic mice, the number of DP and SP are significantly reduced. Numbers below each bar indicate the numbers of mice analyzed. (C) βββαα transgenic mice lack mature T cells. The percentage of mature T cells in thymus, lymph nodes and spleens was determined by immunofluorescence analysis with anti-CD3ε and anti-TCRβ mAbs. A systemic reduction of mature αβ T cells was found in βββαα transgenic mice. Numbers below each bar indicate the numbers of mice analyzed. (D) Two-color immunofluorescence analysis with CD4/CD8 or CD45R/TCRβ specific mAbs. Typical examples of non-transgenic FVB control mice and transgenic βββΔ and βββαα mice are shown. Whereas the numbers and subsets of immature and mature αβT cells are comparable in non-transgenic FVB control mice and transgenic βββΔ mice, transgenic mice expressing the βββαα chain have a systemic reduction of mature T cells. Numbers in each quadrant indicate percentages.

pressed 5.6 ± 0.9% (n = 6), a value significantly lower than the 13.8 ± 0.7% observed in nontransgenic FVB mice. However, the frequency of Vβ6+ T cells was not significantly different in βββαα transgenic mice 10.6 ± 0.7% (n = 6) compared to the FVB control mice (9.8 ± 0.6%, n = 28).

Cell Surface Expression of the Mutant TCR Chains. Extremely high levels of βββΔ and βββ-PI chains were found on the surface of thymocytes of βββΔ (Fig. 5) and βββ-PI transgenic mice (not shown), as determined by flow cytometry. Low, but detectable levels of the βββαα chain were found on the βββαα transgenic thymocytes and peripheral T cells (Fig. 5). The latter also expressed high levels of endogenous TCR/CD3 complexes (Fig. 3 D). The products encoded by the βααββ and βαααα transgenes could not be detected at the surface of thymocytes and mature T cells as determined by flow cytometry with a Vβ8-specific mAb (data not shown). The failure of the βααββ and βαααα mutant TCR chains to appear on the cell surface could explain the absence of any observable effects on T cell development (Fig. 3) and usage of endogenous TCRβ V-gene segments (Fig. 4).

Disproportional Surface Expression of TCRβ and CD3 Components on βββΔ Thymocytes. Two-color immunofluorescence analysis of normal thymocytes using TCRβ- and CD3ε-specific antibodies demonstrates the characteristic
staining pattern for the $\alpha \beta$TCR/CD3 complex, with proportional levels of TCR$\beta$ and CD3$\varepsilon$ components. Surprisingly, the surface levels of $\beta \beta \beta \alpha$ and CD3$\varepsilon$ are disproportional on thymocytes from $\beta \beta \beta \alpha$ mice (Fig. 6 A). Immature (i.e., CD3 low and CD3 int) and mature (CD3 high) thymocytes were found to express the $\beta \beta \beta \alpha$ protein at unusually high levels.

The composition of the $\beta \beta \beta \alpha$/CD3 complex was further analyzed biochemically (Fig. 6 B). Analysis of CD3$\varepsilon$-specific immunoprecipitates revealed a normal composition of disulfide-linked $\alpha \beta$TCR heterodimers associated with CD3$\delta$, CD3$\gamma$, CD3$\varepsilon$, and disulfide-linked CD3$\xi$ components on thymocytes from non-transgenic FVB, transgenic TCR$\beta$ and $\beta \beta \beta \alpha$ mice. Beside normal levels of functional TCR/CD3 complexes, subsequent immunoprecipitations with anti-V$\beta$8-specific mAbs indicated that $\beta \beta \beta \alpha$ thymocytes express high levels of $\beta \beta \beta \alpha$ monomers, apparently in the absence of CD3 components. This explains the disproportional $\beta \beta \beta \alpha$CD3 ratio found on $\beta \beta \beta \alpha$ transgenic thymocytes.

Anchoring of $\beta \beta \beta \alpha$ Monomers through Glycosyl-phosphatidylinositol. The possibility that anchoring occurred through glycosyl-phosphatidylinositol (GPI) was tested by incubating thymocytes from $\beta \beta \beta \alpha$ transgenic mice, and as a control from $\beta \beta \beta \beta$ transgenic mice, with or without phosphatidylinositol-specific PLC, which can specifically release GPI-linked proteins from the cell surface. Subsequently, the cells were analyzed for surface TCR$\beta$ expression by flow cytometry. Upon PLC-treatment, thymocytes from $\beta \beta \beta \alpha$ transgenic mice revealed a staining pattern, characteristic for thymocytes from control FVB mice and TCR$\beta$ transgenic mice (Fig. 7), indicating that the $\beta \beta \beta \alpha$ monomers appear in a GPI-linked form at the cell surface.

T Cell Development in RAG-1-deficient Mice Expressing the Mutant TCR$\beta$ Chains. To study the effects of the mutant TCR chains on T cell development in the absence of endogenous TCR$\alpha$ or TCR$\beta$ chains, we introduced the mutant transgenes into the V(D)J recombination-deficient RAG-1$^{-/-}$ background (5). The $\beta \beta \beta \alpha$ chain appears at the cell surface and was found to efficiently induce thymocyte development, as indicated by CD4/CD8 induction and expansion of the T cell pool to wild-type numbers (Table 1 and Fig. 8). Like the $\beta \beta \beta \beta$ chain, the $\beta \beta \beta$-PI chain does appear at the cell surface but does not permit development of CD4$^{+}$CD8$^{-}$ thymocytes into CD4$^{+}$CD8$^{+}$ thymocytes in the RAG-1$^{-/-}$ background, indicating that surface expression of the extracellular TCR$\beta$ domains in the absence of the transmembrane region cannot promote this step in thymocyte development. Thymocytes of $\beta \beta \beta \alpha$ transgenic RAG-1$^{-/-}$ mice expressed low but significant levels of the $\beta \beta \beta \alpha$ chain on the cell surface (Fig. 8 B). Interestingly, 7% of the thymocytes from $\beta \beta \beta \alpha$ transgenic RAG-1 mutant mice had a CD4$^{+}$CD8$^{-}$ phenotype. Thus, the $\beta \beta \beta \alpha$ chain is able to induce the transition from CD4$^{+}$CD8$^{-}$ to CD4$^{+}$CD8$^{+}$ thymocytes causing the formation of a small but significant CD4$^{+}$CD8$^{+}$ thymocyte subset (Fig. 8 A). However, the $\beta \beta \beta \alpha$ chain fails to increase the number of thymocytes (Table 1). The $\alpha \alpha \beta \beta$ and the $\beta \alpha \alpha \alpha$ chains do not appear at the cell surface and do not promote thymocyte maturation (Fig. 8).
Figure 6. High levels of $\beta_{88\Delta}$ monomers appear at the surface of immature thymocytes in the absence of CD3 components. (A) Proportional surface expression of CD3e and TCRβ on thymocytes from $\beta_{88\Delta}$ transgenic mice. The unusually high levels of $\beta_{88\Delta}$ chains on thymocytes from $\beta_{88\Delta}$ transgenic mice are compared to CD3e levels by two-color immunofluorescence analysis. Thymocytes from nontransgenic controls and $\beta_{88\Delta}$ transgenic mice were stained with FITC-conjugated anti-CD3e (145-2C11) and biotinylated anti-TCRβ (H57-597) mAbs. Biotinylated H57-597 was detected with PE-conjugated streptavidin. Numbers in each quadrant indicate percentages. (B) SDS-PAGE analysis of sequential anti-CD3e and anti-TCRβ immunoprecipitates from surface iodinated thymocytes from nontransgenic FVB mice, transgenic $\beta_{88\Delta}$ and $\beta_{88\Delta}$ mice. Thymocytes from nontransgenic FVB mice (lanes 1), transgenic TCRβ (lanes 2) and $\beta_{88\Delta}$ mice (lanes 3) were surface iodinated and lysed in digitonin containing lys buffer (11). The composition of the CD3e and TCRβ associated components of the $\alpha$TCR/CD3 complex were analyzed by sequential immunoprecipitations with CD3e and Vβ specific mAbs. After three precleaning steps with normal mouse serum (3rd preclear), CD3e associated TCR/CD3 components were immunoprecipitated by two sequential immunoprecipitations with anti-CD3e (1st CD3e and 2nd CD3e). Any residual anti-CD3e mAbs were precipitated by an additional preclear step with protein A-Sepharose beads (Prot. A). Subsequently any residual $\beta_{88\Delta}$ chains and associated molecules were precipitated with F23.1 mAbs indirectly coupled by rabbit anti-mouse IgG to protein A beads (anti-Vβ). Immunoprecipitates were analyzed under non-reducing and reducing conditions. Molecular mass markers are indicated. The 25-30-kD species and bands larger than 60 kD found in all Vβ-specific immunoprecipitations are Ig-light and Ig-heavy chains from contaminating B cells.

Discussion

Previously, we have demonstrated that a mutant TCRβ chain lacking the variable domain (Δββββ) can induce thymocytes to differentiate from the CD4-CD8- to the CD4+CD8+ stage (10, 26). To identify the regions within the TCRβ constant region required for this step in thymocyte development, we have extended the mutational approach by generating five additional TCRβ transgenic mice in which constant region domains were deleted or exchanged by corresponding regions of the TCRα chain. Although all mutant TCR transgenes are highly expressed at the mRNA and protein level, the $\beta_{88\Delta}$ and the $\beta_{88\Lambda}$ chimeric chains could not be detected at the cell surface of thymocytes and peripheral T cells. Both proteins share a variable TCRβ domain and the extracellular constant region of the TCRα chain, including the connecting peptide which contains the cysteine residue responsible for the covalent interchain bond with TCRα. The structure of the $\beta_{88\Delta}$ and $\beta_{88\Lambda}$ chains might not allow assembly with endogenous TCR and CD3 components in the ER and subsequent transport to the cell surface (48). The failure of $\beta_{88\Delta}$ and $\beta_{88\Lambda}$ chains to appear at the cell surface can explain the lack of any noticeable effects on thymocyte development in $\beta_{88\Delta}$ and $\beta_{88\Lambda}$ transgenic mice, on both wild-type and RAG-1−/− backgrounds.

The $\beta_{88\Lambda}$ chimeric chain resembles more closely a TCRβ chain than the $\beta_{88\Delta}$ and $\beta_{88\Lambda}$ chain. The
Figure 8. (A) Induction of CD4 and CD8 expression on thymocytes of mutant TCR transgenic RAG-1-deficient thymocytes. Thymocytes isolated from wild-type (WT), RAG-1-deficient (RAG-1-/-), and the five different transgenic RAG-1-deficient mice (RAG-1-/- x mutant TCRβ transgene) were double stained using CD4- and CD8-specific mAb and analyzed by flow cytometry. Numbers below each dot plot indicate the absolute number of double-positive thymocytes. (B) Surface expression of mutant TCRβ chains on RAG-1-deficient thymocytes. Thymocytes from control RAG-1-deficient (solid lines) and mutant TCRβ transgenic RAG-1-deficient mice (dashed lines) were stained with H57-597 conjugated to FITC or PE and analyzed by flow cytometry. Shown are representative experiments. All experiments were repeated at least four times and gave consistent results. Similar data were obtained when cells were stained with F23.1 (anti-TCRβ).

Table 1. Cellularity, Pre-T Cell Induction and Surface Expression in the Thymus of RAG-1 Mutant Mice Reconstituted with Mutant TCR Transgenes

| Transgene V C CP TM CT No. of thymocytes No. of DP cells Surface expression |
|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| WT                   | β β β β β β β           | 1.4 X 10^8             | 1.3 X 10^8             | Yes                   |
| RAG-1 NA             | β β β β β β β           | 2 X 10^6               | 2.5 X 10^3             | NA                    |

Δ, Domain deleted; * PI, Linked via Q7 b PI sequence.

βββαα chain, which contains the extracellular cysteine required for covalent heterodimerization with TCRα and likely also with the pTα chain, appears at very low levels at the cell surface, as indicated by flow cytometric analysis. This suggests that the βββαα chain participates in the TCR/CD3 complex. However, the βββαα chain cannot fully substitute for the function of the TCRβ chain in promoting differentiation to CD4+CD8+ cells and in mediating allelic exclusion. The latter was indicated by the reduced usage of the Vβ2 gene segment in mature T cells of βββαα transgenic mice. Mature thymocytes expressing high βββαα TCR/CD3 levels were not detected. Apparently, βββαα thymocytes depend on a non-mutant endogenous TCRβ chain to mature into single positive thymocytes.

To specifically analyze the role of βββαα in the transition of thymocytes from the CD4-CD8- to the CD4+CD8+ stage we determined the capacity of the βββαα chain to mediate thymocyte differentiation in βββαα transgenic, RAG-1-/- mice. Expression of the βββαα chain induced the developmental transition from CD4-CD8- to CD4+CD8+ cells as indicated by the presence of a low but significant number of CD4+CD8+ thymocytes in the compound mutant mice, whereas such cells were not found in non-transgenic RAG-1-/- mice. Thus, the βββαα chain, although expressed at low levels at the surface of thymocytes, probably as a component of the preTCR/CD3 complex, can induce the expression of CD4 and CD8 receptors.
The βββΔ transgene functions in most aspects as a normal ββββ transgene as judged by thymocyte numbers, size of the different CD4/CD8 subsets and induction of pre-T cell development in βββΔ:RAG-1−/− mice. Allelic exclusion in βββΔ transgenic mice appears to be efficient as indicated by the virtual absence of T cell subsets expressing rearranged endogenous VB2 and VB6 gene segments in these mice. Thus, the cytoplasmic tail of the TCRβ chain is dispensable for its role in these aspects of T cell development. Remarkably, beside normal surface levels of disulfide linked TCRα/ββββ heterodimers associated with CD3 components, ββββ chains appear at high levels as GPI bound monomers in the absence of CD3 components on the surface of thymocytes and B cells (data not shown). Thymocytes are known to regulate their surface levels of TCR/CD3 complexes posttranslationally. Single subunits or partial complexes that are synthesized in excess are retained in the ER and degraded (49). Previous studies have shown that retention of individual subunits of oligomeric receptors is controlled by ER retention signals located in the cytoplasmic domain. A di-lysine motif at position −3 and −4 or −5 from the carboxyl terminus has been shown to control receptor assembly by retaining unassembled individual chains in the ER (50, 51). The cytoplasmic tails of TCRβ proteins harbor such a di-lysine ER retention signals, VRKKNS in Cβ1 and VKKKNS in Cβ2. Indeed, ββββ chains can escape ER retention, TCR/CD3 assembly and proteolytic degradation.

In normal TCRβ chains GPI linkage might be prevented by the cytoplasmic tail. Therefore, the cytoplasmic tail of the TCRβ chain which is encoded by a separate exon appears to be essential for determining the early processing events of unassembled TCRβ chains rather than being required for TCR/CD3 assembly and signaling thymocyte differentiation.

When compared to nontransgenic littermates, the βββα and the βββΔ transgenes were found to interfere with the usage of the endogenous VB2 segment in the αβT cell lineage. In all founder lines analyzed, the βββα and the βββΔ transgenes consistently excluded endogenous VB2-gene segments more efficiently than endogenous VB6-gene segments. This phenomenon may relate to the position of the V segment in the TCRβ locus (52). The VB2 gene segment is the most 5′ VB segments, distal of the Dβ/Jβ clusters whereas the VB6 segment is located proximal to the Dβ/Jβ clusters. The suggested processivity of the recombination machinery from 3′ to 5′ would more profoundly affect VB genes lying distal from the DJ join (53–56). Alternatively, pre-T cell development induced by functional TCRβ chains might signal the shut down of endogenous TCRβ loci from 5′ to 3′, resulting in the preferential rearrangement of VB-segments lying proximal to the DJ region.

In conclusion, we have identified the extracellular constant domains and the transmembrane region of the TCRβ chain to be required for initiating pre-T cell development as demonstrated by the efficient allelic exclusion of endogenous TCRβ alleles in RAG-1−/− mice and transition of CD4+CD8− to CD4+CD8+ thymocytes in βββΔ-transgenic RAG-1−/− mice. Allelic exclusion and pre-T cell development is inefficient in transgenic mice expressing the βββα chain. Both the extracellular constant region and the transmembrane region of the TCRβ chain, but not the variable domain and cytoplasmic tail, are required for its assembly with the other TCR/CD3 components. The cytoplasmic tail of the TCRβ chain appears to be essential for retention of the TCRβ chain in the ER, but not for signal transduction. The finding that only those mutants that theoretically can form disulfide bonds to the pTα chain and assemble with CD3 components into a pre-TCR complex can catalyze the transition from CD4−CD8− to CD4+CD8+ cells supports the notion that cell surface expression of the preTCR/CD3 complex is a prerequisite for pre-T cell differentiation (29).

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