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 $CD4^+8^+$ 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 (pTα) 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 pTα gene in mice demonstrated the crucial role of preTα 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-

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sition from $CD4^-CD8^-$ thymocytes to $CD4^+CD8^+$ thymocytes, concomitant with all the changes described for recombination-deficient mice reconstituted with a functional TCRβ transgene or the mutant ΔV-TCRβ transgene (see above). Thus, cross-linking of the CD3 modules on $CD4^-CD8^-$ thymocytes apparently mimics the signaling mediated by the pre-TCR complex.

Earlier experiments have shown that the variable domain of the TCRβ 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 TCRβ transgenic mice with defined alteration in the TCRβ constant region.

Materials and Methods

Construction of Mutant TCR Transgenes

Starting material for the construction of the ββββΔ, βββαα, ββααββ, and βαααα mutants (see Fig. 1) was a 20-kb Asp 718 (KpnI) TCRβ fragment from the cosmid clone cosHYβ9-1.14-5 (9). Mutations were restricted to the 6.1-kb BamHI fragment containing all the four exons of the Cβ2 constant region; transcriptional control elements such as the TCRβ promoter and the 3′ TCRβ enhancer region as well as the functional VDJ region were not altered.

Construction of a Mutant TCRβ Transgene Lacking the Cytoplasmic Tail (ββββΔ). The ββββΔ protein was generated by converting the 2nd codon of the 4th TCRβ 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 $C\beta_2$ BamHI fragment, second a 1.940-kb PstI-HpaI fragment and third a 322-bp EcoRV fragment containing the TCRβ constant exon 4 encoding the putative cytoplasmic tail of the TCRβ chain was cloned into pSP72. By recombinant PCR the second codon AAG was mutated into a TAG stop codon, which introduces a XbaI restriction site (TCTAGA).

Two recombinant PCR primers, anti-sense (as) and sense (s), were used in combination with T7- and SP6-specific primers, respectively. Primer T7, 5′-AAATTAATACGACTCACTATAGGG3′. Primer SP6, 5′-ATACACATACGATTTAGGTGACAC-3′. Primer as, 5′-C TCAGGAATTTTTTTTCTAGACCTGAGAAAGAGA-AGA C-3′. Primer s, 5′-GTCTTCTCTTTCTCAGGTCTA-GAAAAAAAATTCCTGAG-3′.

PCR products were digested with XbaI and EcoRV restriction endonucleases yielding a 199-bp and a 123-bp XbaI/EcoRV fragment. These fragments were cloned into the polycloning sites of the pSP73 and pSP72 vectors respectively (Invitrogen, San Diego, CA). These plasmids were digested with XbaI and BglI and the appropriate fragments were ligated to generate a pSP72/73 hybrid vector containing the mutated 322-bp EcoRV fragment. Presence of the mutation was verified by sequencing. The reinsertion of the mutated 322-bp EcoRV fragment into the 20-kb Asp718 fragment was analogous to the subcloning procedure described above.

Construction of Mutant TCRα/β Chimeric Transgenes βββαα, ββααββ, βαααα. To generate the βαααα TCR chimeric gene, a 6.1-kb BamHI fragment containing the complete TCRβ constant region (Cβ2) was replaced by the 8.6-kb BamHI genomic fragment from the cosmid clone cos H-Yα9.1-36 which contains the TCRα constant region (Cα). Orientation was determined by ClaI and EcoRI digestions (see physical maps in references 9, 32). For the ββααββ and βββααα TCR chimeric genes, a 5′ and a 3′ fragment of the Cβ2 and Cα fragments were cloned into pSP72 or pSP73 respectively (Invitrogen). The 5′ fragments contain exon 1 and 2, and the 3′ fragments contain exon 3 and 4 of the TCR constant regions. These plasmids were digested with PvuI and partially with BglII and the appropriate fragments were ligated to obtain the 8.2-kb 5′Cβ-3′Cα and the 6.5-kb 5′Cα-3′Cβ hybrid-fragments flanked by BamHI sites. This step introduces a BglII-EcoRV-ClaI-EcoI linker from the polycloning site of the pSP plasmids. Replacement of the 6.1-kb Cβ2 BamHI fragment from TCRβ with the 5′Cβ-3′Cα and 5′Cα-3′Cβ BamHI hybrid-fragments generates the βββββα and βααββ TCR transgenes, respectively. A 200-bp BamHI fragment 5′ of the 6.1-kb Cβ2 intron fragment was deleted at this step. Orientation was determined by ClaI digestion.

Construction of the βββ-PI Transgene. Starting material for this mutant transgene was a 20-kb KpnI fragment harboring the functional TCRβ gene of the encephalomyelitogenic T cell clone cloned into λ Charon 39 (33). In brief, the TCRβ insert was digested to completion with BamHI. Three of the resulting restriction fragments were then subcloned into pKS(-) (Stratagene); a 8.3-kb BamHI fragment containing the functionally rearranged VDJβ (pTCRβ 8.3), a 7.3-kb BamHI fragment containing the genomic Cβ2 region (pTCRβ 7.3) and a 4.7-kb BamHI-KpnI fragment containing the 3′end of the phage clone (p4.7 kb). Site-directed mutagenesis was performed on the pTCRβ 7.3 subclone to introduce an AccI restriction site 39 bp 3′ of the start of the TM exon of Cβ2 by using oligonucleotide-directed in vitro mutagenesis (Amersham Corp., Arlington Heights, IL); the resulting clone was called pCβ2Acc1. A 2.3-kb BamHI-SacI fragment from pTCRβ 7.3 containing the first exon of Cβ2 (Ig-like region), a 687-bp SacI-AccI fragment from pCβAcc1 containing the 2nd exon (encoding the connecting peptide) of Cβ2 and the first 13 aa of exon 3 of Cβ2 encoding part of the TM region, and a 1.4-kb AccI-BamHI fragment from Q72-6-220ApBG (a gift from Dr. Gerry Waneck, Massachusetts General Hospital, Boston, MA) containing the $Q7^b$ PI anchor signal and SV40 poly adenylation signal was ligated into BamHI digested pKS(-) to generate $pC\beta_2PI$. To construct the mutated TCRβ transgene used for injection a trimolecular ligation was performed with the 8.3-kb BamHI fragment from pTCRβ 8.3, the 4.4-kb BamHI fragment $pC\beta_2PI$, and BamHI linearized dephosphorylated p4.7 BK plasmid. The resulting construct $pTgC\beta_2PI$ was checked for orientation of the respective fragments relative to each other by restriction mapping. An ~17-kb NotI-KpnI restriction fragment was prepared from $pTgC\beta_2PI$ and used for the production of transgenic mice.

Transgenic Mice

Fertilized mouse oocytes were recovered in cumulus from the oviducts of superovulated females that had mated with FVB males several hours earlier. The DNA-fragments (2–4 μg/ml 10 mM Tris-HCl/0.1 mM EDTA [pH 7.5]) were injected into the most accessible pronucleus of each fertilized egg essentially as described (34). After overnight culturing, 2-cell stage embryos were implanted into the oviducts of day 1 pseudopregnant C57Bl/6 foster animals and carried to term. Several weeks after birth total genomic DNA was prepared from tail biopsies as described (35). Transgenic founders were crossed with FVB mice. To exclude insertion specific phenotypic effects, mice derived from three to six different founder lines were analyzed for each construct. The generation of TCRβ (βββββ) transgenic mice has been described (9).

DNA and RNA Blot Analysis

For Southern blot analysis 10 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 ^{32}P-labeled probes and washed as described (36). Probes used for DNA analysis were Vβ8.1 (37) and Jβ2 (38). The final wash was at 0.1× SSC, 60°C and 0.1× 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), Cα (40), Cβ (41), and actine (42) were ^{32}P-labeled by random priming, hybridization conditions were as described (36) with the addition of 1% SDS to all solutions. The final wash was at 0.1× 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 βββββ, ββββΔ, and βββ-PI transgenic mice (10^6/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)[1] (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 Lysis 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 × 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 Iscoves medium supplemented with Con A and 10% FCS. After 3 d, activated lymphocytes were isolated on a density gradient. B cells were depleted with sheep anti-mouse Ig coupled to magnetic beads, according to the manufacturers procedure (Advanced Magnetics, Cambridge, MA). Enriched T lymphocytes were washed twice and starved for 1 h in methionine-free RPMI-medium (Selectamine kit; GIBCO BRL, Gaithersburg, MD) supplemented with 10% dialyzed serum and counted. Lymphocytes (20–30 × 10^6) were labeled metabolically for 4 hours with 100 μCi [^{35}S]methionine in 1 ml methionine-free RPMI-medium in a humidified incubator at 37°C, 5% CO_2. Subsequently the cells were harvested and lysed for 30 min at 4°C in NP-40 containing lysis buffer (50 mM triethanolamine-Cl, pH 7.5, 5 mM $MgCl_2$, 1 mM PMSF, 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).

[1] *Abbreviations used in this paper*: GPI, glycosyl-phosphatidylinositol; PLC, phospholipase C

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/LiA mice were crossed with FVB mice. The T cell compartments in TCRβ transgenic FVB × C57Bl/LiA F1 and C57Bl/LiA 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

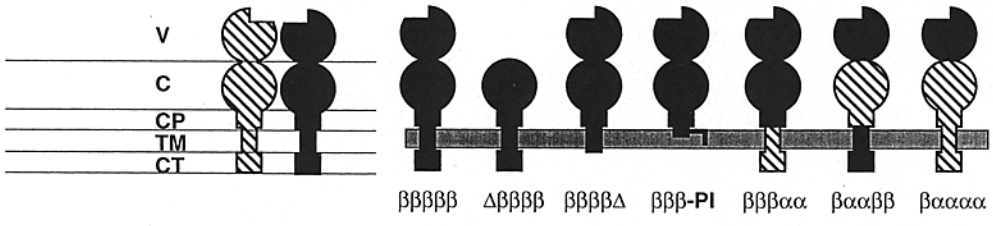

Figure 1. Schematic illustration of the proteins encoded by the mutant TCR transgenes. TCRα protein (ααααα, *dashed*) and TCRβ protein (βββββ, *black*) share a common basic 5-domain structure: V, variable domain; Ig, Ig-like domain; CP, connecting peptide, TM, transmembrane region; CT. cytoplasmic tail. Beside the non-mutant TCRβ transgene and the mutant Δβββββ chain, five additional alterations were introduced into the TCRβ constant regions. The ββββΔ mutant resembles a TCRβ protein lacking its cytoplasmic tail and the βββ-PI mutant comprises the three extracellular constant regions of the TCRβ chain and the first 13 aa of the TCRβ transmembrane region connected to the PI-anchor sequence of Q7[b]. The ββββαα, 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.

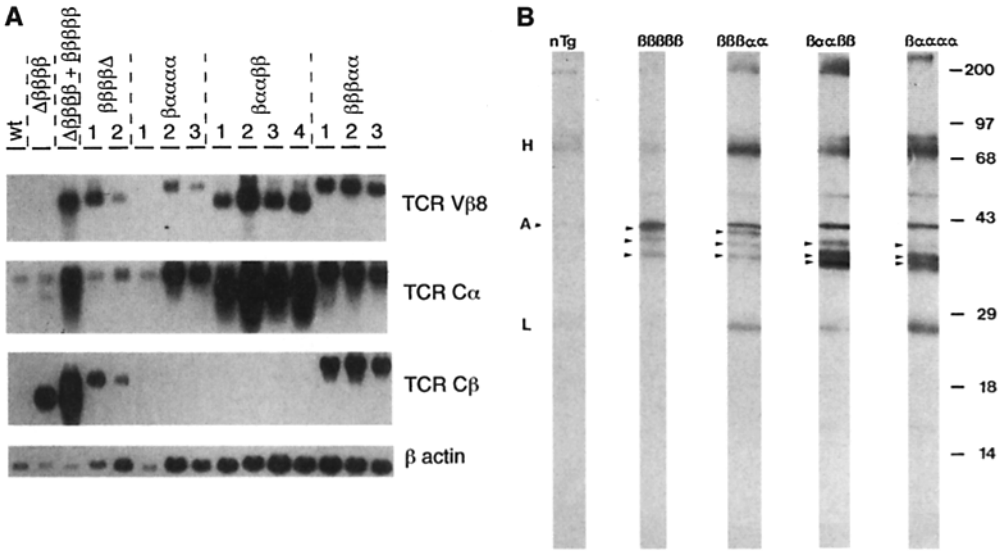

Figure 2. Expression of the mutant TCR transgenes. (*A*) Northern blot analysis to study expression of the mutant TCR transgenes. Total RNA extracted from spleens of different mutant TCR founder lines was analyzed for the presence of transgene specific transcripts RNA samples from non-transgenic FVB mice (*wt*), Δβββββ transgenic mice and βββββΔ/Δβββββ double-transgenic mice are included as controls. Northern blots were hybridized with Vβ8-, constant TCR Cα-, and TCR Cβ-specific probes (*B*) SDS-PAGE analysis of anti-Vβ8 immunoprecipitates from metabolically labeled Con A blasts of mutant TCR transgenic mice. Vβ8-specific immunoprecipitates from NP40 lysates of metabolically labeled Con A blasts derived from splenocytes of a non-transgenic FVB mouse (*nTg*) and mutant TCR transgenic mice were analyzed under reducing conditions by SDS-PAGE analysis on a 10–15% gradient gel. Arrow heads indicate the different glycosylation forms of the mutant TCR chains. Contaminating immunoglobulin heavy and light chain (*H* and *L*) are due to indirect precipitations with rabbit anti mouse Ig. *A* indicates the position of actin. The position of molecular weight markers are indicated (kD).

in the other regions. As expected, βββαα and βαααα transcripts co-migrated at the position of mature TCRα transcripts (1.5 kb) whereas the βββββΔ and βααββ transcripts run at the position of mature TCRβ transcripts (1.3 kb). The size of the βββ-PI mRNA was also in accordance with the structure of the βββ-PI transgene (not shown). Hybridization patterns with ^{32}P-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 VB8-specific immunoprecipitates from NP40-lysates of metabolically labeled Con A activated splenocytes. T cell enriched splenocytes from non-transgenic FVB mice and transgenic βββββ, βββαα, βααββ, 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 Vβ8-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 βββαα 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 found in different mutant transgenic lines. With the exception of βββαα transgenic mice, the absolute number of thymocytes in the mutant transgenic lines was comparable to that of non-transgenic mice (Fig. 3 *A*). In βββαα 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 βββαα 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 βββαα transgenic mice was normal, the number of the CD4$^+$CD8$^+$, CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ subsets were clearly reduced, suggesting that the βββαα 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 βββαα 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 βββββΔ 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 βββββΔ 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 βββββΔ transgene prevented surface expression of rearranged Vβ2- and Vβ6-gene segments was 99% and 83%, respectively.

In βββαα transgenic FVB mice, the Vβ2-subset com-

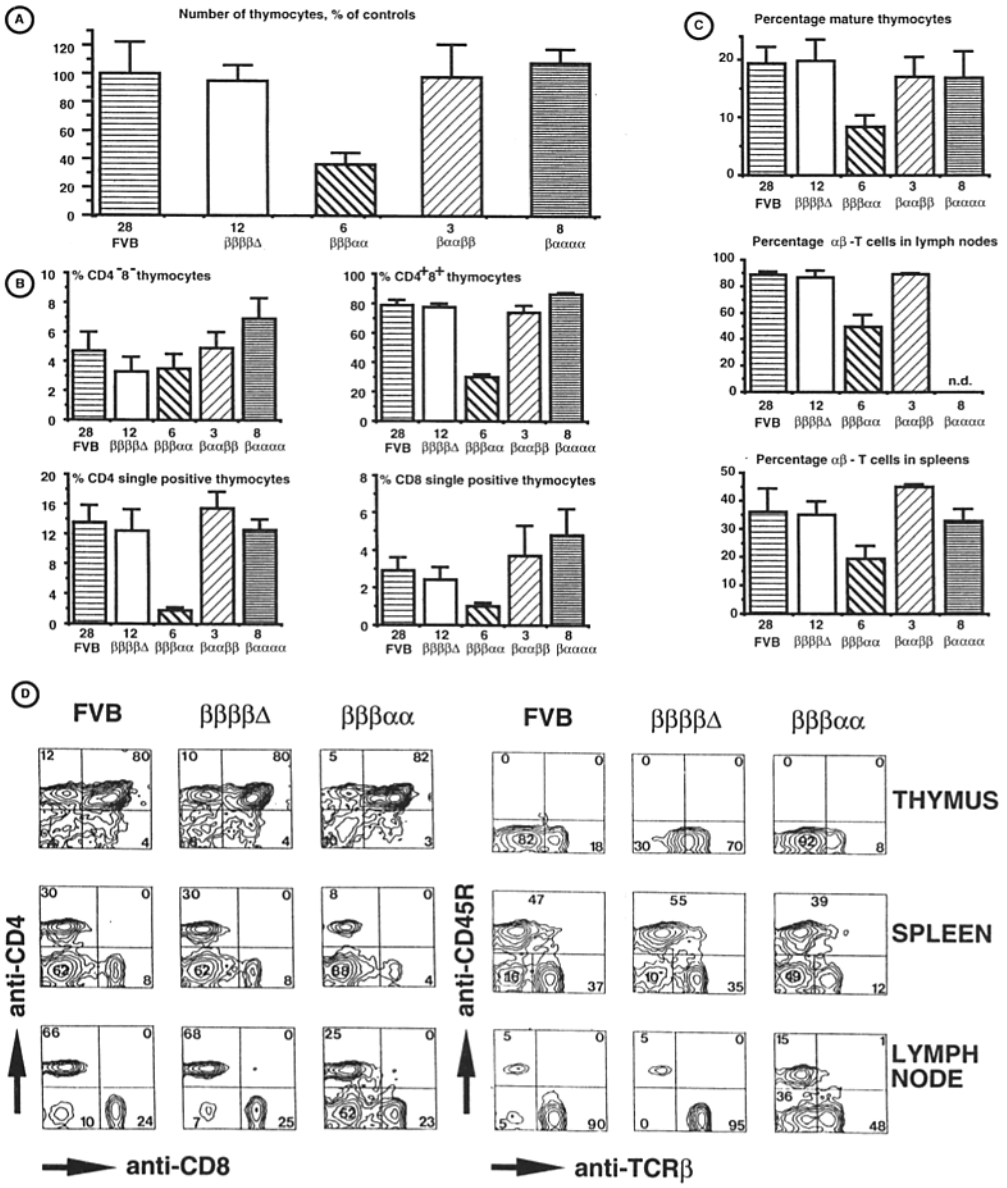

Figure 3. Quantiative comparison of the αβ T cell compartment in mutant TCR transgenic mice. (*A*) Relative number of thymocytes in mutant TCR transgenic mice. The number of thymocytes was determined by flow cytometry on a Sysmex F800 and compared to nontransgenic littermates and/or age matched FVB mice. In ββββαα 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 littermates. 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 $CD4^+CD8^-$ or $CD4^-$ $CD8^+$ 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 Lysis II software (Becton Dickinson). Although the number of DN pro-thymocytes is normal in ββββαα transgenic mice, the number of DP and SP are significantly reduced. Numbers below each bar indicate the numbers of mice analyzed. (*C*) ββββαα transgenic mice lack mature T cells. The percentage of mature T cells in thymus, lymphnodes 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 ββββαα transgenic mice. Numbers below each bar indicate the numbers of mice analyzed (*D*) Two-color immunofluorescence analysis of thymus, spleen and lymph nodes with CD4/CD8 or CD45R/TCRαβ specific mAbs. Typical examples of non-transgenic FVB control mice and transgenic βββββΔ and ββββαα mice are shown. Whereas the numbers and subsets of immature and mature αβT cells are comparable in non-transgenic FVB control mice and transgenic βββββΔ mice, transgenic mice expressing the ββββαα chain have a systemic reduction of mature T cells Numbers in each quadrant indicate percentages.

prised 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 $V\beta6^+$ T cells was not significantly different in ββββαα 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 βββββΔ and βββ-PI chains were found on the surface of thymocytes of βββββΔ (Fig. 5) and βββ-PI transgenic mice (not shown), as determined by flow cytometry. Low, but detectable levels of the ββββαα chain were found on the surface of ββββαα transgenic thymocytes and peripheral T cells (Fig. 5). The latter also express 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 βββββΔ Thymocytes. Two-color immunofluorescence analysis of normal thymocytes using TCRβ- and CD3ε-specific antibodies demonstrates the characteristic

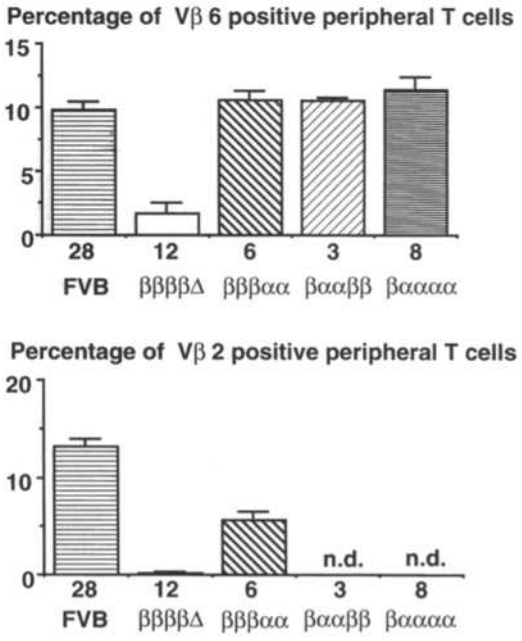

Figure 4. Frequencies of peripheral T cell subsets in mutant TCRβ transgenic mice expressing Vβ2- and Vβ6- domains. The relative size of the Vβ2- and Vβ6- T cell subsets was determined by two-color flow cytometry and corrected for the percentage of mature T cells, as determined by anti-CD3ε and anti-TCRβ staining. The number of mice analyzed is indicated below each bar; nd, not determined. Mice from three to six different founder lines were included per mutant TCR construct.

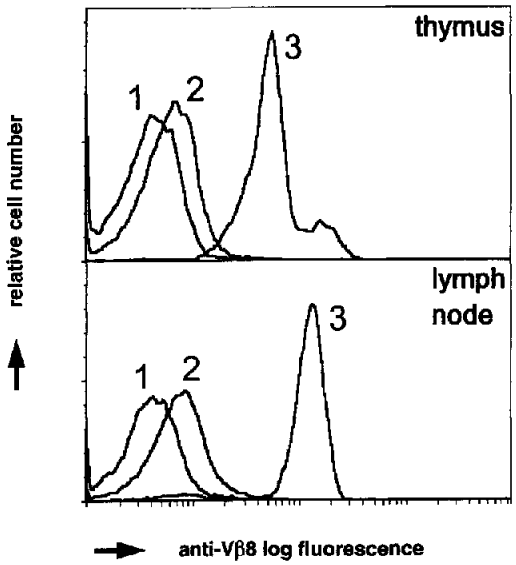

Figure 5. Surface expression of mutant TCR chains. Thymocytes and lymphnode cells from non-transgenic FVB control mice (*1*) and transgenic βββαα (*2*) and βββββΔ (*3*) mice stained with transgene-specific FITC conjugated anti-Vβ8 mAb.

staining pattern for the αβTCR/CD3 complex, with proportional levels of TCRβ and CD3ε components. Surprisingly, the surface levels of βββββΔ and CD3ε are disproportional on thymocytes from βββββΔ mice (Fig. 6 *A*). Immature (i.e., $CD3^{low}$ and $CD3^{int}$) and mature ($CD3^{high}$) thymocytes were found to express the βββββΔ protein at unusually high levels.

The composition of the βββββΔ/CD3 complex was further analyzed biochemically (Fig. 6 *B*). Analysis of CD3ε-specific immunoprecipitates revealed a normal composition of disulfide-linked αβTCR heterodimers associated with CD3δ, CD3γ, CD3ε, and disulfide-linked CD3ζ components on thymocytes from non-transgenic FVB, transgenic TCRβ and βββββΔ mice. Beside normal levels of functional TCR/CD3 complexes, subsequent immunoprecipitations with anti-Vβ8-specific mAbs indicated that βββββΔ thymocytes express high levels of βββββΔ monomers, apparently in the absence of CD3 components. This explains the disproportional βββββΔ/CD3 ratio found on βββββΔ transgenic thymocytes.

Anchoring of βββββΔ Monomers through Glycosyl-phosphatidylinositol. The possibility that anchoring occurred through glycosyl-phosphatidylinositol (GPI) was tested by incubating thymocytes from βββββΔ transgenic mice, and as a control from βββββ 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 TCRβ expression by flow cytometry. Upon PLC-treatment, thymocytes from βββββΔ transgenic mice revealed a staining pattern, characteristic for thymocytes from control FVB mice and TCRβ transgenic mice (Fig. 7), indicating that the βββββΔ monomers appear in a GPI-linked form at the cell surface.

T Cell Development in RAG-1-deficient Mice Expressing the Mutant TCRβ Chains. To study the effects of the mutant TCR chains on T cell development in the absence of endogenous TCRα or TCRβ chains, we introduced the mutant transgenes into the V(D)J recombination-deficient $RAG\text{-}1^{-/-}$ background (5). The βββββΔ 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 βββββΔ chain, the βββ-PI chain does appear at the cell surface but does not permit development of $CD4^-CD8^-$ thymocytes into $CD4^+CD8^+$ thymocytes in the $RAG\text{-}1^{-/-}$ background, indicating that surface expression of the extracellular TCRβ domains in the absence of the transmembrane region cannot promote this step in thymocyte development. Thymocytes of βββαα transgenic $RAG\text{-}1^{-/-}$ mice expressed low but significant levels of the βββαα chain on the cell surface (Fig. 8 *B*). Interestingly, 7% of the thymocytes from βββαα transgenic RAG-1 mutant mice had a $CD4^+CD8^+$ phenotype. Thus, the βββαα 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 βββαα chain fails to increase the number of thymocytes (Table 1). The βααββ and the βαααα chains do not appear at the cell surface and do not promote thymocyte maturation (Fig. 8).

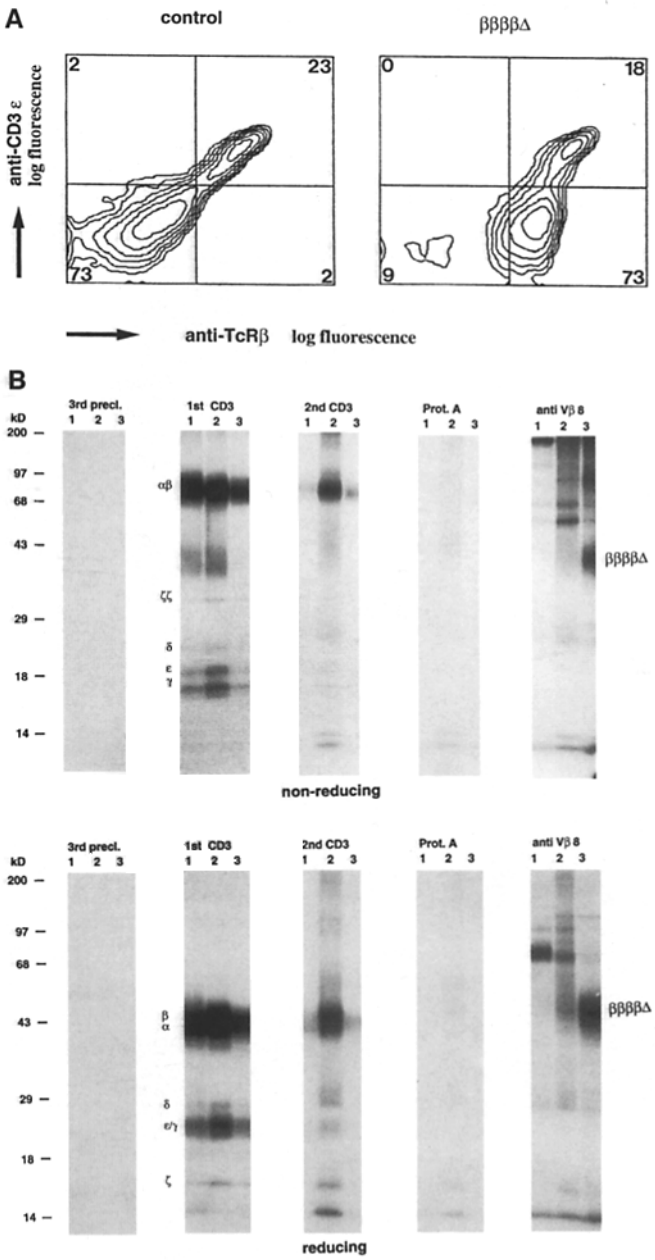

Figure 6. High levels of ββββΔ monomers appear at the surface of immature thymocytes in the absence of CD3 components. (*A*) Disproportional surface expression of CD3ε and TCRβ on thymocytes from ββββΔ transgenic mice. The unusually high levels of ββββΔ chains on thymocytes from ββββΔ transgenic mice are compared to CD3ε levels by two-color immunofluorescence analysis. Thymocytes from nontransgenic (*control*) and ββββΔ transgenic mice were stained with FITC-conjugated anti-CD3ε (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-CD3ε and anti-Vβ8 immunoprecipitates from surface iodinated thymocytes from nontransgenic FVB mice, transgenic ββββββ and ββββΔ mice. Thymocytes from nontransgenic FVB mice (lanes *1*), transgenic TCRβ (lanes *2*) and ββββΔ mice (lanes *3*) were surface iodinated and lysed in digitonin containing lysis buffer (11). The composition of the CD3ε and TCRβ associated components of the αβTCR/CD3 complex were analyzed by sequential immunoprecipitations with CD3ε and Vβ8 specific mAbs. After three preclearing steps with normal mouse serum (3rd preclear), CD3ε associated TCR/CD3 components were immunoprecipitated by two sequential immunoprecipitations with anti-CD3ε (1st CD3ε and 2nd CD3ε). Any residual anti-CD3ε mAbs were precipitated by an additional preclear step with protein A–Sepharose beads (Prot. A). Subsequently any residual ββββΔ chains and associated molecules were precipitated with F23.1 mAbs indirectly coupled by rabbit anti–mouse IgG to protein A beads (anti-Vβ8). 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 Vβ8-specific immunoprecipitations are Ig-light and Ig-heavy chains from contaminating B cells.

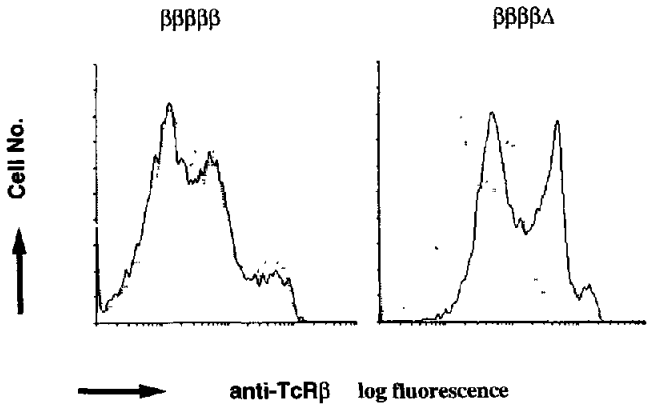

Figure 7. Release of ββββΔ chains from the surface of ββββΔ thymocytes by phosphatidylinositol-specific phospholipase C (PLC). PLC-treated (+PLC, *dashed curve*) and mock-treated (−PLC, *solid curve*) thymocytes from ββββββ and ββββΔ transgenic mice (10^6/ml) were washed twice, stained with FITC-labeled anti-TCRβ antibodies, and analyzed with FACScan® (Becton Dickinson).

Discussion

Previously, we have demonstrated that a mutant TCRβ chain lacking the variable domain (Δββββββ) 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 βααββ and the βαααα 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 TCRβ. The structure of the βααββ and βαααα 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 βααββ and βαααα chains to appear at the cell surface can explain the lack of any noticeable effects on thymocyte development in βααββ and βαααα transgenic mice, on both wild-type and RAG-$1^{-/-}$ background.

The ββββαα chimeric chain resembles more closely a TCRβ chain than the βααββ and βαααα chain. The

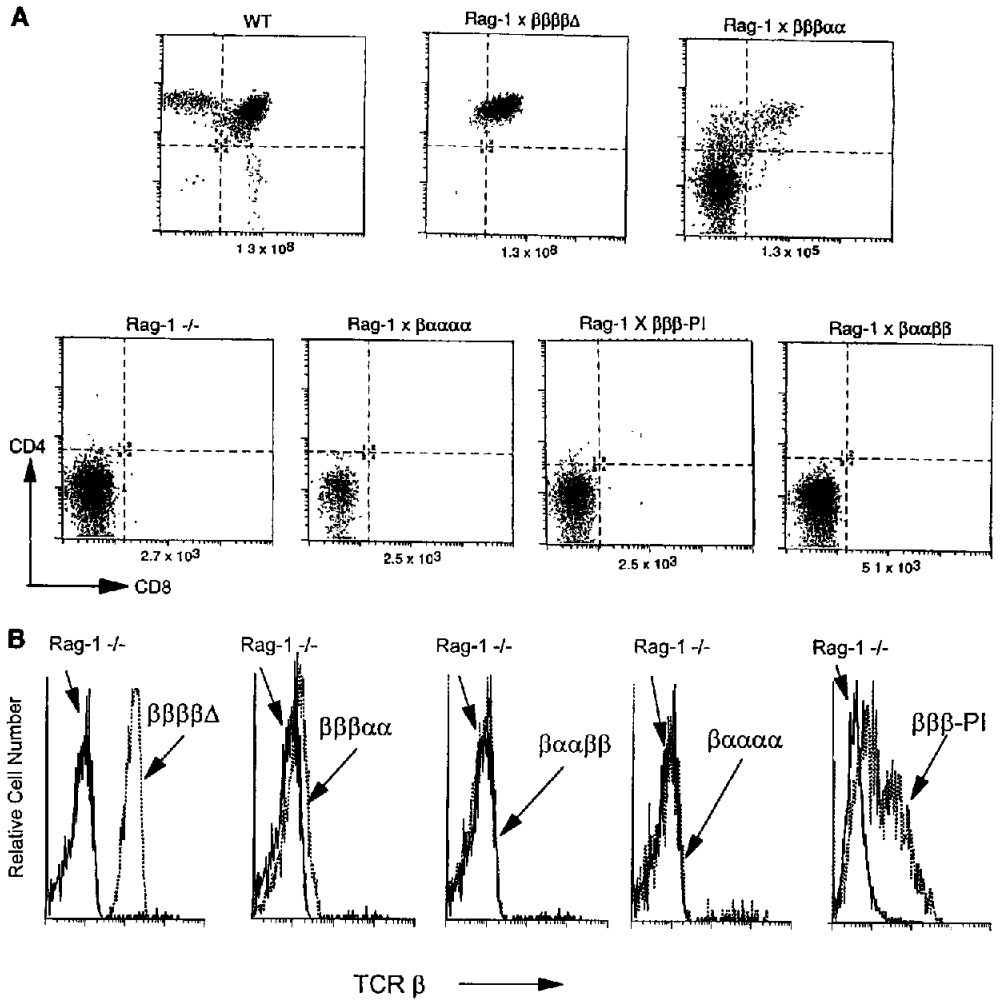

Figure 8. (*A*) Induction of CD4 and CD8 expression on thymocytes of mutant TCR transgenic RAG-1-deficient thymocytes. Thymocytes isolated from wild-type (*WT*), RAG-1-deficient (RAG-$1^{-/-}$) and the five different transgenic RAG-1-deficient mice (RAG-$1^{-/-}$ × mutant TCRβ transgene) were double 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-Vβ8).

ββββαα 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 ββββαα chain participates in the TCR/CD3 complex. However, the ββββαα 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 ββββαα transgenic mice. Mature thymocytes expressing high ββββαα TCR/CD3 levels were not detected. Apparently, ββββαα thymocytes depend on a non-mutant endogenous TCRβ chain to mature into single positive thymocytes.

To specifically analyze the role of ββββαα in the transition of thymocytes from the $CD4^-CD8^-$ to the $CD4^+$ $CD8^+$ stage we determined the capacity of the ββββαα chain to mediate thymocyte differentiation in ββββαα transgenic, RAG-$1^{-/-}$ mice. Expression of the ββββαα 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 ββββαα 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.

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	Total No. of thymocytes	Absolute No. of DP cells	Surface expression
ββββΔ	β	β	β	β	Δ	1.4×10^8	1.3×10^8	Yes
ββββαα	β	β	β	α	α	1.9×10^6	1.3×10^5	Yes
βααββ	β	α	α	β	β	3.7×10^6	5.1×10^3	No
βαααα	β	α	α	α	α	7×10^5	2.5×10^3	No
βββ-PI	β	β	β	PI		2×10^6	2.5×10^3	Yes
WT	β	β	β	β	β	1.6×10^8	1.3×10^8	Yes
RAG-1	NA					2×10^6	2.5×10^3	NA

Δ, Domain deleted, ‡PI, Linked via $Q7^b$ PI sequence.

The ββββΔ transgene functions in most aspects as a normal ββββββ transgene as judged by thymocyte numbers, size of the different CD4/CD8 subsets and induction of pre-T cell development in ββββΔ;RAG-$1^{-/-}$ mice. Allelic exclusion in ββββΔ transgenic mice appears to be efficient as indicated by the virtual absence of T cell subsets expressing rearranged endogenous Vβ2 and Vβ6 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/ββββΔ heterodimers associated with CD3 components, ββββΔ 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, VKRKNS in Cβ1 and VKKKNS in Cβ2. Indeed, ββββΔ 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 βββαα and the ββββΔ transgenes were found to interfere with the usage of the endogenous Vβ2 segment in the αβT cell lineage. In all founder lines analyzed, the βββαα and the ββββΔ transgenes consistently excluded endogenous Vβ2-gene segments more efficiently than endogenous Vβ6-gene segments. This phenomenon may relate to the position of the V segment in the TCRβ locus (52). The Vβ2 gene segment is the most 5′ Vβ segments, distal of the Dβ/Jβ clusters whereas the Vβ6 segment is located proximal to the Dβ/Jβ clusters. The suggested processivity of the recombination machinery from 3′ to 5′ would more profoundly affect Vβ 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 Vβ-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 ββββΔ-transgenic RAG-$1^{-/-}$ mice. Allelic exclusion and pre-T cell development is inefficient in transgenic mice expressing the βββαα 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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