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Involvement of the TCR Cβ FG Loop in Thymic Selection and T Cell Function

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Abstract

The asymmetric disposition of T cell receptor (TCR) Cβ and Cα ectodomains creates a cavity with a side-wall formed by the rigid Cβ FG loop. To investigate the significance of this conserved structure, we generated loop deletion (βΔFG) and βwt transgenic (tg) mice using the TCR β subunit of the N15 CTL. N15βwt and N15βΔFG H-2b animals have comparable numbers of thymocytes in S phase and manifest developmental progression through the CD4+CD8+ double-negative (DN) compartment. N15βΔFG facilitates transition from DN to CD4+8+ double-positive (DP) thymocytes in recombinase activating gene (RAG)-2−/− mice, showing that pre-TCR function remains. N15βΔFG animals possess twofold more CD8+ single-positive (SP) thymocytes and lymph node T cells, consistent with enhanced positive selection. As an altered Vα repertoire observed in N15βΔFG mice may confound the deletion's effect, we crossed N15αβ TCR tg RAG-2−/− with N15βΔFG tg RAG-2−/− H-2b mice to generate N15αβ, RAG-2−/− and N15αβ, βΔFG RAG-2−/− littermates. N15αβ, βΔFG RAG-2−/− mice show an 8–10-fold increase in DP thymocytes due to reduced negative selection, as evidenced by diminished constitutive and cognate peptide-induced apoptosis. Compared with N15αβ, N15αβ, βΔFG T cells respond poorly to cognate antigens and weak agonists. Thus, the Cβ FG loop facilitates negative selection of thymocytes and activation of T cells.

Key words: negative selection • thymocyte development • TCR structure • Ig superfamily • transgenic mice

Introduction

The TCR is a multicomponent complex composed of a T disulfide-linked heterodimer in noncovalent association with the CD3 subunits (1–5). The majority of T cells express a T-encoded by α and β TCR genes (Tαβ) whereas a numerically minor but functionally significant subset expressed Tα encoded by γ and δ TCR genes (Tγδ; for a review, see reference 6). The Tαβ heterodimer whose overall shape and architecture are similar to an antibody Fab fragment represents the peptide–MHC complex (pMHC)* ligand binding unit. Thus, Tαβ determines the ligand specificity of an individual T cell. In contrast, the CD3 polypeptides mediate TCR–based signal transduction. The signaling function of the CD3 components involves a conserved motif, termed immunoreceptor tyrosine-based activation motif (ITAM), present at 1–3 copies in the cytoplasmic domain of each CD3 subunit (7–9). The various CD3 subunits exhibit different interactions with intracellular signaling factors and, acting in concert, lead to T cell activation (10–15).

The TCR complex contains one copy of CD3γ and CD3β and two copies of CD3ε and CD3ζ configured as two CD3γζ and CD3ζε heterodimers and a disulfide-linked CD3ζ homodimer (16–21). Recent crystallographic analysis (22) in conjunction with epitope mapping of a class I–restricted TCR (23) have identified a cavity within the Tαβ constant (C) module of sufficient size to accommodate one nonglycosylated CD3ε ectodomain. In this regard, the solution structure of a heterodimeric CD3γζ
ectodomain complex reveals a unique side-to-side hydrophobic interface between the two C2-set immunoglobulin-like domains and indicates that a modular pair-wise association is shared between CD3εδ as well (24). CD3ε has an elongated shape with length, width and depth being ~40, 25, and 25 Å, respectively.

Herein, we have investigated the biological significance of the unusual structural arrangement of the CB FG loop that forms the side wall of this C-module cavity. Mapping studies using the CB FG loop–specific mAb H57 previously showed that one of two CD3ε subunits resides adjacent to this structural element. To this end, we have created TCR transgenic mice bearing either intact or CB FG loop–deleted β chains. Our results show that the loss of the CB FG loop attenuates negative selection without blocking positive selection within the thymus at the level of double-positive (DP) thymocytes. The sensitivity of mature T cells to cognate peptides as well as altered peptide ligands is also reduced. In contrast, pre-TCR facilitated transition from CD44+CD25+ to CD44+CD25− double-negative (DN) thymocytes and subsequent DP thymocyte generation is not altered. The implications of these findings for TCR and pre-TCR function are discussed.

Materials and Methods

The following mAbs were used: R-phycoerythrin (PE)– or Cyochrome-conjugated anti–mouse CD4 (H129.19); FITC– or Cyochrome-conjugated anti–mouse CD8α (53–6.7); Cyochrome-conjugated anti–TCR CB (H57–597); PE-conjugated anti–CD25 (PC61); FITC-conjugated anti–CD44 (IM7); FITC-conjugated anti–Vβ5.1, 5.2 (MR9.4); biotin–conjugated anti–CD69 (H.1.2F3); biotin–conjugated anti–Vα2 (B20.1), anti–Vβ3.2 (RR3–16), anti–Vα8.3 (B21.14), anti–Vα11.1, 11.2 (RR8–1), PE-conjugated streptavidin (BD Pharmingen). For flow cytometry, single cell thymocyte or lymph node suspensions were prepared in PBS containing 2% FCS and 0.05% NaN3. Cells were stained at 5 × 106 cells per ml in PBS–2% FCS and 0.05% NaN3, containing the antibodies at saturating concentrations. Phenotypes and proportions of thymocyte and lymph node cell subsets were analyzed by two-color flow cytometry using a FACScan™ (Becton Dickinson) and the CELLQuest™ program. Dead cells were excluded from the analysis by forward and side scatter gating.

Bromodeoxyuridine Staining. Bromodeoxyuridine (BrdU) incorporation was examined according to the manufacturer’s protocol (BrdU Flow Kit; BD Pharmingen). Mice were administered two intraperitoneal injections of 1 mg of BrdU in PBS at 4 h intervals. After 18 h, thymocytes were fixed/permeabilized and stained with FITC-conjugated anti-BrdU mAb following treatment with 30 μg/ml DNase. Live cells were gated and analyzed on a FACScan™.

Apoptosis Assay. Thymocytes from untreated, VSV8 peptide-injected (0.5 μg intravenously, 18 h previously) or dexamethasone-treated (0.5 mg intraperitoneally, 6 h previously) were examined. Apoptosis of thymocytes was evaluated by cellular DNA content determined by propidium iodide (PI) staining and alterations in plasma membrane permeability examined by Annexin V staining. For PI staining, after fixation with 40% ethanol at 4°C for 30 min, total thymocytes (106 cells) were resuspended in PBS containing 50 μg/ml RNase A and 2.5 μg/ml PI and incubated at 37°C for 30 min. PI incorporation was analyzed by flow cytometry. For annexin V staining, after washing with PBS, total thymocytes (106 cells) were resuspended in 1× binding buffer containing 0.5 μg/ml FITC-conjugated annexin V (ApoAlert Annexin V Apoptosis kit; CLONTECH Laboratories,
Inc.) and 2.5 μg/ml PI to gate out dead cells, and incubated in the dark for 10 min at room temperature, followed by the analysis on a FACScan™.

Proliferation Assay. Lymph node cells from N15αβ or N15αβ−βΔFG mice (10^5/well) were incubated at 37°C with 2 × 10^4 irradiated EL-4 thymoma cells, which had been preloaded for 2 h with the indicated doses of VSV8 or a weak agonist, L4, in AIM-V medium (Life Technologies) containing 50 μM 2-ME. After 48 h incubation, 0.4 μCi per well of [3H]TdR (ICN Biomedicals) was added, and after an additional 18 h of culture at 37°C, the cells were harvested and the incorporated radioactivity was measured.

Measurement of IFN-γ. IFN-γ production was induced under the same culture conditions used for proliferation assays (see above). After 48 h of incubation, supernatants were collected and assayed for IFN-γ using a mouse IFN-γ ELISA kit (Mouse IFN-γ OptEIA Set; BD PharMingen). The sensitivity of the assay was 31.3–2,000 pg/ml and results were calculated as the mean of duplicate wells.

Results

The TCR Cβ FG Loop Structure and Sequence Comparison. One remarkable feature of the TCR Cβ domain is the striking elongation of the FG loop. Compared with other Ig-like structures (aside from the TCR Cγ domain), there is a 13 aa insertion within the FG loop (27). Fig. 1 A shows a view of the crystallographically resolved murine N15 class I MHC-restricted αβ TCR (Vβ5.2 D2β2.6 Cβ2/Vα8 Jα5Co; reference 22). The unique protrusion of the Cβ domain’s FG loop is apparent (bracketed in a black box). It was previously noticed that internally the loop is well structured (22). The Trp225 at the tip of the loop

Figure 1. Structure and sequence analysis of the TCR Cβ FG loop region. (A) Structure of the N15αβ-TCR heterodimer. The Va and VB variable domains and the Cα and Cβ constant domains are labeled and the strands of Cβ designated. The uniquely protruding FG loop of Cβ is bracketed in a black box. Cysteine residues forming intradomain disulfide bonds in each of the four domains are shown in yellow and the four glycosylation sites discussed in the text are colored in light blue with labels. (B) A close up view of the region in the black box with the FG loop and the A and B strands labeled. The view is re-oriented relative to panel A to best highlight critical side chains. Note that W225, L219, and P232 of the FG loop pack onto L119 and V122 to form a mini-hydrophobic core. See text for a detailed description of this rigid entity. For clarification, other side chains are not shown with the exception of cisP154 at the beginning of the BC loop. Relevant hydrogen bonds are indicated as dashed lines. Graphics were generated using SETOR (reference 62). (C) Sequence comparison of the TCR Cβ FG loop regions from various species. The position of the F and G strands is defined based on the above crystallographic analysis. The shaded region defines the 14 aa residue deleted from the N15 TCR Cβ chain. Boxed residues mark the potential N-linked glycosylation signals. The β chain cysteine (247) which forms an interchain disulfide with the α chain, is indicated by the semi-solid circle and the F strand cysteine (212) which forms the intradomain disulfide with the β strand cysteine (147) of Cβ (not shown) is indicated by the solid circle.
plays a key role. A hydrogen bond between the NE1 of Trp225 and the carbonyl group of Ser229 brings the bulky indole ring of Trp225 in position to make hydrophobic contacts with Leu219 and Pro232 at the base of the FG loop (Fig. 1 B, a close up view of the local region shown in the black box of Fig. 1 A). The hydrophobic patch composed of these three FG loop residues packs onto Leu119 and Val122 at the beginning of the CB domain’s A strand to form a mini-hydrophobic core. The Leu119 and Val122 themselves lie on a stable local strut created by a tight turn between Asp118 and Asn121. Moreover, a pair of mainchain hydrogen bonds between Thr123 on the A strand and Phe153 at the end of the B strand further stabilizes the local structure. Collectively, the CB domain FG loop, albeit a long protrusion, is an integrated component of a rigid structural entity that connects the VB and CB domains. Not only are the residues mentioned above all invariant among mouse, rat, human, chimpanzee, rabbit, cow, dog, and pig as shown in the Fig. 1 C, but the three-dimensional structural feature depicted in Fig. 1 B is also conserved in all known TCR crystal structures, including class I MHC-restricted N15 and 2C from mouse (22, 28) and B7 from human (29), as well as the human class II MHC-restricted HA1.7 (30).

Figure 2. Altered phenotypes of T lineage cells in the N15βΔFG mice. (A) TCR expression on CD8+ SP lymph node T cells in the N15βwt and N15βΔFG mice. Lymph node cells from 8-wk-old N15βwt and N15βΔFG mice were triple-stained with anti-CD4, anti-CD8, and anti-TCR. CB FG loop specific H57 or anti-Vβ5 MR9.4 mAbs. The histograms of TCR, CB FG loop (left) and Vβ5 (right) expression on the gated CD8+ SP lymph node T cells are shown. The numbers represent the percentages of H57- or Vβ5-positive cells (as indicated). (B) The CD4/CD8α double-staining profiles in thymocytes and lymph node cells are altered by the lack of the TCR CB FG loop. The thymocytes and lymph node cells from N15βwt and N15βΔFG mice at 8 wk of age were double-stained with PE-anti-CD4 and FITC-anti-CD8α. The expression of CD4 (x axis) and CD8α (x axis) on thymocytes and lymph node cells was detected by flow cytometry after gating on 10,000 live cells. The percentages of each subset are indicated.

Furthermore, as shown by the representative CD4/CD8α double-staining profiles in Fig. 2 B, there is a detectable increase in the percentage of the CD8+ SP subset and a decrease in that of the DP subset in N15βΔFG thymocytes compared with N15βwt mice. Therefore, N15βΔFG mice show a statistically significant increase in the absolute number of CD8+ SP thymocytes and a concomitant decrease in DP thymocytes (Table I). This increase in CD8+ SP cells is further reflected by the increase in CD8+ SP lymph node T cells (N15βwt, 1.99 ± 0.77 × 10^7 [n = 10]; N15βΔFG 3.45 ± 0.82 × 10^7 [n = 15]). From these findings, we conclude that the absence of the FG loop in the constant region of N15 TCR β chain influences T cell development in the tg mouse model.

There are two major checkpoints at which αβ thymocyte development is dependent upon the TCR β chain. The earliest occurs during the transition from DN to DP thymocytes and is regulated by the pre-TCR complex...
Table I. Absolute Numbers of Total and Cell Subsets of Thymocytes and LN Cells in N15βwt and N15βΔFG Mice

|            | Thymus | LN       |
|------------|--------|----------|
|            | Total  | DP       | CD8+ SP | CD4+ SP | DN      | Total | CD8+ SP | CD4+ SP |
| N15βwt     | 12.05 ± 2.99 | 9.95 ± 2.47 | 0.60 ± 0.17 | 1.08 ± 0.30 | 0.41 ± 0.16 | 6.36 ± 2.11 | 1.99 ± 0.77 | 0.49 ± 0.19 |
| N15βΔFG    | 9.51 ± 2.09 | 7.31 ± 1.61 | 0.85 ± 0.29 | 1.02 ± 0.27 | 0.33 ± 0.12 | 7.17 ± 1.83 | 3.45 ± 0.82 | 0.71 ± 0.21 |

*P < 0.05.
1P < 0.01.
2P < 0.0005.
3The numbers of cell subsets in thymocytes and lymph node T cells were calculated by quantifying the total number of thymocytes and lymph node cells from 6–12-wk-old N15β (n = 10) and N15βΔFG (n = 15) mice and the percentages of each subset as determined by FACS® analysis. Results are expressed as mean ± SD (×10⁶ cells). Statistical differences between N15βwt and N15βΔFG numbers in indicated populations are given.

Figure 3. Early thymocyte development is unaffected by the absence of TCR Cβ FG loop.
(A) Normal proliferation/turnover in N15βΔFG mice. 9-wk-old N15βwt and N15βΔFG mice were injected intraperitoneally twice at 4 h intervals with 1 mg of BrdU in PBS at 4 h intervals, and 18 h later, thymocytes were stained with FITC-anti-BrdU mAb and assayed by flow cytometry. The histograms of BrdU incorporation on 10,000 live thymocytes are shown. The numbers represent the percentages of BrdU-positive cells. (B) The transition from CD44+/CD25+ to CD44−/CD25− DN thymocytes is not blocked in N15βΔFG mice. Total thymocytes were triple-stained with FITC-anti-CD44, PE-anti-CD25, and Cyochrome-anti-CD4/anti-CD8α. The profiles of CD44 (y axis) versus CD25 (x axis) expression in Cyochrome-negative (CD4−/CD8−) DN thymocytes are shown. The percentage of each subset is indicated. (C) Efficient generation of DP thymocytes in N15βΔFG RAG-2−/− mice. Thymocytes from 8-wk-old N15βΔFG RAG-2−/− mice were double-stained with PE-anti-CD4 and FITC-anti-CD8α. The expression of CD4 (y axis) and CD8α (x axis) on thymocytes was detected by flow cytometry after gating on 10,000 live cells. The percentages of each subset are indicated.

Comprised of the invariant pTα subunit disulfide linked to a TCR β chain in noncovalent association with the CD3 subunits (34). The later checkpoint involves the αβ TCR and occurs during the transition from DP to SP thymocyte maturation. At that time, self-MHC–restricted, nonautoreactive T cells are selected for maturation and peripheral exportation (35). To investigate the mechanism of the altered thymocyte phenotype in N15βΔFG mice, we first compared early thymic development in the N15βwt and N15βΔFG mice by examining the fraction of cells synthesizing DNA using BrdU incorporation in vivo. In this way, it is possible to test whether the FG loop deletion in the N15 TCRβ chain affects proliferation and/or turnover of thymocytes. The N15βwt and N15βΔFG mice were injected twice intraperitoneally with 1 mg of BrdU in PBS at 4 h intervals, and 18 h later, thymocytes were assayed for the incorporation of BrdU by flow cytometry. As shown in Fig. 3 A, the percentage of BrdU+ thymocytes in the N15βΔFG mice was comparable to that observed in control N15βwt mice, indicating that the number of cells that had progressed through S phase is similar. In addition, we compared the developmental stages of immature thymocytes by examining the surface expression of CD25 and CD44 on DN thymocytes of these mice. The percentages of the four CD44+/CD25−, CD44+/CD25+ , CD44−/CD25−, and CD44−/CD25+ in the N15βΔFG were equivalent to those of N15βwt mice (Fig. 3 B), suggesting that the deletion of Cβ FG loop from the N15 TCRβ chain does not disrupt the pre-TCR–facilitated transition from CD44+/CD25+ to CD44−/CD25− DN thymocytes. To further assess the function of the Cβ FG loop–deletion in pre-TCR signaling at the CD44+/CD25+ DN stage, we established RAG−2−/− mice expressing the Cβ/CD25− DN stage in the RAG−2−/− mice, leading to strong proliferation of DN thymocytes and efficient generation of DP cells (36). As shown in Fig. 3 C, N15βΔFG RAG-2−/− mice generate DP thymocytes with essentially normal cellularity...
leading to the alterations in thymic and peripheral lymphocytes. The FG loop exerts an influence on selection of the TCR repertoire.

Table II. Vα Usages by CD8+ SP and CD4+ SP Lymph Node Cells in N15βΔFG Mice

| Vα Usage | CD8+ SP | CD4+ SP |
|----------|---------|---------|
|          | Va2     | Va3.2   | Va8.3  | Va11 |          | Va2   | Va3.2 | Va8.3 | Va11 |
| N15βwt   | 12.21 ± 0.95d | 1.13 ± 0.43 | 3.74 ± 0.54 | 5.46 ± 0.50 | 11.77 ± 1.38 | 1.90 ± 0.46 | 2.00 ± 0.59 | 5.74 ± 1.54 |
| N15βΔFG  | 12.19 ± 0.75 | 0.99 ± 0.38 | 4.37 ± 0.58 | 6.81 ± 0.70 | 13.61 ± 0.95 | 1.77 ± 0.58 | 2.35 ± 0.48 | 4.74 ± 1.04 |

*p < 0.02.
np < 0.00005.
pp < 0.005.
dLymph node cells from 6-12-wk-old N15β (n = 10) and N15βΔFG (n = 15) mice were stained with FITC anti-CD4, CyChrome anti-CD8, biotin anti-Vα mAb, followed by PE-conjugated streptavidin. Values represent the mean and SD of percentages in CD8+ and CD4+ SP lymph node T cell subsets as determined by FACS® analysis.
on thymocytes was detected by flow cytometry after gating on 10,000 live cells. The percentages of each subset are indicated. (C) The N15αβ,βΔFG mice show a drastic increase in the total and DP thymocytes, but a decrease in the CD8+ SP lymph node T cells. The numbers of total, DP, CD8+ SP, and DN thymocytes and CD8+ SP lymph node T cells were calculated by quantifying the total numbers of thymocytes and lymph node cells from 8–12-wk-old N15αβ and N15αβ,βΔFG mice and the percentages of each subset as determined by FACS® analysis. Open circles (N15αβ mice [n = 13]) and closed circles (N15αβ,βΔFG mice [n = 9]) represent values of individual mice and bars represent average values for a given group. The total and DP thymocyte numbers are significantly higher, and CD8+ SP lymph node cell number is significantly lower in the N15αβ,βΔFG mice, compared with the N15αβ mice (total and DP thymocytes, P < 0.0001; CD8+ SP lymph node cells, P < 0.0005).

Decreased Thymic Negative Selection in the N15αβ,βΔFG Mice. The T thymocyte repertoire is shaped by both negative and positive thymic selection processes acting primarily at the level of the immature DP thymocytes (for reviews, see references 35 and 38). Previous studies showed that DP thymocytes are deleted during negative selection via a caspase-dependent apoptotic mechanism (39). To investigate the basis for the dramatic increase in DP thymocytes in N15αβ,βΔFG, we evaluated the efficiency of the negative selection process in the N15αβ versus N15αβ,βΔFG animals. Apoptotic cells were enumerated through measurement of DNA content in total thymocytes using propidium iodide and flow cytometric analysis. As shown in Fig. 5 A, the percentage of thymocytes with subdiploid DNA content, a typical feature of apoptotic cell death, is significantly higher in the N15αβ mice than in the N15αβ,βΔFG mice (N15αβ, 12.5% versus N15αβ,βΔFG, 1.1%). Consistent with this result, alterations in plasma membrane permeability, one of the earliest changes in cells undergoing apoptosis, is also altered differentially in N15αβ versus N15αβ,βΔFG mice. As shown in Fig. 5 B, about half (48.5%) of the thymocytes in the N15αβ mice are positive for annexin V staining, suggesting that a large number of DP thymocytes are being continuously deleted by apoptosis. In contrast, only a small percentage (10.0%) of thymocytes are annexin V positive in N15αβ,βΔFG mice. This finding suggests that the nature of constitutive ongoing negative selection in DP thymocytes is different between N15αβ and N15αβ,βΔFG mice.

To further probe differences in the negative selection threshold of DP thymocytes from N15αβ and N15αβ,βΔFG mice, we injected the cognate peptide VSV8 intravenously and compared the phenotypic changes in thymocytes. 18 h after intravenous injection with 0.5μg of VSV8 peptide, N15αβ mice showed an ~90% reduction in total thymocyte number (without VSV8 injection, 2.07 × 10^7 cells; after VSV8 injection, 0.26 × 10^7 cells), consistent with earlier observations (25). By contrast, there is no peptide-induced change in the total thymic cellularity in N15αβ,βΔFG mice (without VSV8 injection, 10.4 × 10^7 cells; after VSV8 injection, 10.6 × 10^7 cells). These re-
results suggest that deletion of the Cβ FG loop alters the sensitivity of immature DP thymocytes to negative selection induced by the cognate peptide in N15 TCRtg mice. Consistent with the thymic cellularity data, DNA staining with propidium iodide shows a significant increase in the percentage of subdiploid DNA-containing cells after VSV8 injection in the N15αβ (35.8%) but not in the N15αβΔFG mice (1.3%; Fig. 5 A). Furthermore, as shown in Fig. 5 B, the N15αβ mice display a prominent increase in the annexin V+ thymocytes after 18 h of VSV8 (80.4%), compared with N15αβΔFG mice (14.7%). Collectively, these results suggest that the increase in DP thymocytes in the N15αβΔFG mice can be explained, at least in part, by the reduced sensitivity of those DP thymocytes to undergo peptide-triggered negative selection. Although not shown, higher doses of VSV8 peptide induce apoptotic cell death in both N15αβ and N15αβΔFG thymocytes to a similar extent, suggesting that the N15αβΔFG mice require a larger number of pMHC-ligated TCRs to trigger negative selection. That dexamethasone-induced DNA fragmentation of thymocytes is equivalent in both mouse strains implies no general apoptotic defect in N15αβΔFG animals (data not shown). Note that the observed apoptosis of thymocytes is not secondary to peripheral cytokine production observed in other systems (40) as shown by the rapid kinetics of the N15 TCR tg system and prior parallel FTOC analysis (25, 39).

Impaired Proliferation and Cytokine Production upon Antigen-triggered Stimulation of Naive T Cells in the N15αβΔFG Mice. To examine the role of the Cβ FG loop in mature T cell function, we next tested the antigen responsiveness of N15αβΔFG T cells by culturing lymph node T cells with VSV8 cognate peptide or variant APLs in vitro. To first address whether the Cβ FG loop deletion affects early phases of naive T cell activation, we assessed surface expression of the cell activation “markers,” IL-2Rα (CD25) and CD69, which represents a reliable method for determining the number of T cells that are activated in culture (41, 42). The lymph node T cells from N15αβ and N15αβΔFG mice were stimulated in vitro for 18 h with varying molar concentrations of VSV8 or the APL variants, L4 and K1 (26), using irradiated EL-4 cells as H-2Kb-bearing APCs, and then were assayed for the expression of CD25 and CD69 by flow cytometry. As shown in Fig. 6 A, VSV8 peptide was capable of activating virtually almost the same percentages of T cells at the peptide concentration of 10^{-4}–10^{-5} M in both N15αβ and N15αβΔFG mice. However, a very subtle difference is observed in the activation responses of the N15αβ and N15αβΔFG lymph node T cells after stimulation with a lower concentration of VSV8 (10^{-10} M) and more convincingly with the APL variants, L4 and K1, as assessed by CD69 (Fig. 6 A) and CD25 (data not shown) expression. At peptide concentrations of 10^{-3}–10^{-7} M for L4 and 10^{-4}–10^{-5} M for K1, the percentages of activated T cells from N15αβΔFG mice are slightly higher than those from N15αβΔFG mice; the dose–response curve from N15αβΔFG lymph node T cells after treatment with L4 or K1 peptide is shifted to the right by a factor of ~10. These findings indicate that in the mature lymphoid compartment, the early T cell activation by weak agonist ligation is modestly affected by the CB FG loop deletion as measured by this assay.

We next examined and compared Ag-induced IFN-γ production and proliferative responses of unprimed naive lymph node T cells from N15αβ and N15αβΔFG mice. As shown in Fig. 6 B, when incubated for 48 h with VSV8 pulsed EL-4 cells, both the N15αβ and N15αβΔFG lymph node T cells secrete IFN-γ into the culture supernatant at each peptide concentration tested (10^{-5}–10^{-9} M).
EL-4 cells prepulsed with the weak agonist L4, the N15αβ T cells can produce readily detectable amounts of IFN-γ at a peptide concentration of 10⁻⁴ M. In contrast, N15αβ,βΔFG lymph node T cells cannot induce IFN-γ production after L4 stimulation at any peptide concentration tested (Fig. 6 B). Similarly, as shown in Fig. 6 C, significant differences are also observed in the IL-2–dependent proliferative responses of the N15αβ and N15αβ,βΔFG lymph node T cells after stimulation with antigenic peptides as assessed by [³H]thymidine incorporation. When N15αβ and N15αβ,βΔFG lymph node T cells are cocultured with irradiated EL-4 cells prepulsed with VSV8 for 48 h, the amounts of incorporated [³H]thymidine in N15αβ,βΔFG lymph node T cells are clearly less than those in N15αβ T cells at peptide concentrations from 10⁻⁶ to 10⁻¹⁰ M (Fig. 6 C). In addition, compared with N15αβ T cells, N15αβ,βΔFG lymph node T cells show significantly decreased proliferative responses to L4 stimulation at peptide concentrations from 10⁻⁴ to 10⁻⁶ M, with the dose–response curves from N15αβ,βΔFG lymph node T cells shifted to the right by a factor of 10–100 (Fig. 6 C). Collectively, these results clearly demonstrate that responsiveness to antigenic peptides is impaired in the N15αβ,βΔFG T cells as determined by peptide antigen-specific induction of proliferation and cytokine production.

Discussion

In this study, we generated a TCR β chain mutant (N15βΔFG) in which the entire Cβ FG loop was deleted. The N15βΔFG construct or the normal N15βwt cDNA was then introduced as a transgene into mice and differentiation/function analysis of thymocytes conducted on the H–2b background. While N15βΔFG facilitated pre-TCR activity (Fig. 3, and Table I), our analysis reveals a distinct phenotype with respect to effects of the βΔFG mutation on further thymocyte maturation. N15βΔFG tg mice show a statistically significant increase in CD8⁺ SP thymocytes and lymph node T cells (Table I) in association with alteration of endogenous Vα repertoires (Table II). These results imply that, in balance, enhanced positive selection is favored over negative selection. Given the potential confounding effects of repertoire alteration, we subsequently restricted Vα expression to that of the N15 α subunit on a RAG-2⁻/⁻ background in a second set of animals. Thus, N15αβ TCR tg RAG-2⁻/⁻ and N15βΔFG tg RAG-2⁻/⁻ mice were crossed to generate N15αβ RAG-2⁻/⁻ and N15αβ–βΔFG RAG-2⁻/⁻ littermates. N15αβ,βΔFG tg RAG-2⁻/⁻ mice were striking with regard to an 8–10-fold increase in thymocyte cellularity as a consequence of an increase in DP thymocytes without alteration in cell numbers of DN or CD8⁺ SP compartments (Fig. 4). This increase was due to reduced negative selection with decreased hypodiploid cellular DNA content constitutively as well as following in vivo VSV8 cognate peptide injection in N15αβ,βΔFG tg RAG-2⁻/⁻ compared with N15αβ tg RAG-2⁻/⁻ H–2b mice (Fig. 5). Annexin V staining further confirmed the re-
duced apoptosis in N15αβ.vΔFG RAG-2−/− versus N15αβ RAG-2−/− mice.

At first glance, these data may appear in conflict with earlier studies by Degermann et al. (43), whose findings indicated that a TCR β chain lacking the identical solvent-exposed Cβ FG loop supported normal αβ T cell development in tg mice as well as normal function in cell transfectants. Their β chain was derived from the 14.3.d TCR specific for HA10−149 PR8 influenza hemagglutinin peptide complexed with I-Ee and comprised of Vβ8.2 and Jβ2.1 segments, unlike the N15ΔFG chain derived from the VSV8/Ko-specific N15 CD8+ SP CTL encoded by Vβ5.2 Jβ2.6 segments. In 14.3.d βΔFG tg mice, superantigen-induced proliferation, anti-H-2d CTL effector function, or helper T cell responses for anti-hapten antibody production were essentially normal. However, given that in the 14.3.d βΔFG tg mice, endogenous TCR α subunits pair with the introduced β transgene product, the biologic system can readily overcome any deficiency mediated by the βΔFG deletion. Hence, polyclonal T cell responses are not a sensitive indicator of the functional consequences of the Cβ FG loop deletion. Consistent with this notion, additional studies by the same authors in 14.3.d βΔFG mice show that αβ T cells coexpressing NK1.1 are severely depleted (44). This is particularly noteworthy, as ~80% of NK1.1 T cells express the invariant (Vα14-Jα281) segment required for their development (45). Thus, even in the RAG-2+/− background, a near single TCR specificity is generated with regard to NK1.1 T cells, permitting the effects of the Cβ FG loop deletion to be recognized. Our results, in conjunction with those of Degermann, imply that the Cβ FG loop is functionally significant for immune recognition-based signaling by both conventional αβ T cells and NK1.1 T cells of pMHC and CD1 specificities, respectively.

Prior mAb epitope mapping analysis showed that one of the two CD3e subunits within a TCR complex is in close proximity to the TCR β chain FG loop whereas the second CD3e subunit lies elsewhere. Crystallographic analysis of the N15 TCR αβ heterodimer reveals a striking asymmetry within the constant domain module such that virtually half of the Cβ domain’s ABED surface is exposed, yet uninvolved in Cα domain contacts. This asymmetry creates a cavity beneath the β chain with the Cβ ABED surface as a ceiling and the plasma membrane as the floor. The two protruding Cα loops (CD loop and EF loop) plus one Cα (N185) and two Cβ (N121, N186) glycans form one sidewall while the Cβ FG loop and one Cβ (N236) glycan form the second wall of this cavity. In structural terms, the role of this extensive FG loop is to further rigidify the interface between Vβ and Cβ domains, thereby limiting the lateral movement of Vβ. In addition, careful analysis of the Cβ FG loop in several TCR structures (22, 28, 30, 46) reveals conservation of the three-dimensional structure of the loop, the adjacent Cβ domain and the cave of both pMHC-I- and pMHC-II-restricted TCRRs in human (29, 30) and mouse (22, 28). Such conservation implies an important role for this structural element on both CD4+ and CD8+ SP T cells. By forming a side wall of the cavity into which a CD3ε subunit of one CD3 heterodimer can insert, the Cβ FG loop presumably stabilizes the interaction between CD3ε and the Triβ heterodimer. Not surprisingly, therefore, immunoprecipitation studies using the CD3εγ dimer-specific mAb 7D6 with N15αβ and N15αβΔFG transfectants reveals a markedly weakened association of CD3εγ with the TCR αβ heterodimer and CD3ε in N15αβΔFG T cells (unpublished data). It is further noteworthy that the loop is negatively charged, perhaps influencing the electrostatics of the TCR surface in a manner similar to an N-linked glycan. Antedating the evolution of the FG loop in higher vertebrates, such a glycan localizes to the same region in primitive species (shark, chicken and axo; data not shown). Although the mouse has an adjacent N-linked glycan as well as the elongated FG loop in its TCR structure, both are not essential since the human TCR lacks an N-linked attachment site.

With these details in mind, we note that the TCR is a largely rigid structure: one component consists of the FG loop—“reinforced” Vβ-Cβ extracellular segment associated via an extensive (~2400 Å2) buried surface interface with the Cα domain of the TCRα subunit, a second component. The Cβ-Cα interface contains multiple rigidifying salt bridges. Hence, the Vβ-Cβ-Cα domains form a single conformationally rigid module. This Vβ-Cβ-Cα module in turn is noncovalently associated with the CD3εγ and/or CD3δε heterodimers such that the glycosylated CD3γ and CD3δ subunits extend away from the TCR heterodimer while the two nonglycosylated CD3ε subunits are proximal. NMR structural studies show that CD3ε and CD3γ (and by extension CD3δε) adopt C2-set IgSF folds, forming a stable heterodimeric complex by parallel strand pairing (G strand) and hydrophobic interaction (24). The CD3ε-CD3γ G strand pairing presumably creates a rod-like connector, permitting displacement of transmembrane helices of CD3εγ and CD3δε upon TCR triggering, leading to intracellular kinase-dependent events and resulting in T lineage activation. Removal of the Cβ FG loop, by eliminating one wall of the cave in the Cβ domain would reduce one key component of the rigidifying architecture such that signaling might be impaired. Hence the altered negative selection in DP thymocytes of the N15αβ.vΔFG animals and the reduced capacity of their CD8+ SP lymph node T cells to proliferate to cognate antigens and APL are anticipated sequelae. That polyclonal thymocytes and αβ T cells in βΔFG tg mice (i.e. RAG-2+/−) can overcome this block, implies either that compensatory adaptations in the TCRα subunit or elsewhere readily occur or that additional protein interactions among TCR subunits (for example, within their transmembrane segments) maintain TCR-based signaling even in the absence of FG loop constraint. These possibilities are not mutually exclusive.

The prominent effect of the βΔFG transgene in N15αβ.vΔFG RAG-2−/− mice is the marked inefficiency of negative selection processes compared with that of N15αβ RAG-2−/− mice. Interestingly, the Cβ FG loop deletion impairs negative selection without diminishing the capacity of DP thymocytes to differentiate to CD8 SP thy-
mocytes; hence, the number of CD8\(^+\) SP thymocytes in N15αβ\(\beta\Delta\)FG RAG-2\(^{-/-}\) H-2\(^b\) and N15αβ RAG-2\(^{-/-}\) H-2\(^b\) littermates is identical. In general, pMHC ligands of stronger affinity are negatively selecting while those with weaker affinity for a given TCR are positively selecting (for reviews, see references 47 and 48). Given that the C\(\beta\) FG loop deletion will reduce rigidity and likely attenuate signaling from negatively selecting ligands, this result is not surprising. In contrast, positive selection must not be attenuated below a threshold required for initiating effective survival signaling. As other studies have demonstrated that positive and negative selection are under the control of different signaling pathways (for reviews, see references 38 and 49), this differential concept is entirely tenable. Nevertheless, in N15αβ,\(\beta\Delta\)FG RAG-2\(^{-/-}\) mice, the numbers of CD8\(^+\) SP lymph node T cells are somewhat reduced compared with N15αβ RAG-2\(^{-/-}\) mice on the same H-2 background. This observation in conjunction with increased HSA and PNA staining of CD8\(^+\) SP thymocytes in N15αβ,\(\beta\Delta\)FG RAG-2\(^{-/-}\) mice (data not shown) suggests that positive selection may not be entirely unaffected by the C\(\beta\) FG mutation.

The pre-TCR consists of a TCR\(\beta\) chain disulfide-bonded to the thymus-restricted p\(\alpha\) subunit expressed on DN thymocytes in association with CD3\(\epsilon\) heterodimers and CD3\(\xi\) homodimers (34, 50). Although the single Ig-like ectodomain of p\(\alpha\) bears only 12\% homology to TCR C\(\alpha\), key residues participating in the formation of the rigid interface between C\(\alpha\) and C\(\beta\) are preserved (22). This structural conservation suggests that the heterodimeric interface of p\(\alpha\)-C\(\beta\) is similar to the interface of C\(\alpha\)-C\(\beta\). From this conserved feature, we have inferred that a CD3\(\epsilon\) heterodimer slots into a pre-TCR cave comparable to that created within the TCR. That a CD3\(\epsilon\) knockout mutation disrupts p\(\alpha\) function (51) is consistent with this view. Furthermore, given that a CD3\(\gamma\) knockout also disrupts pre-TCR function (52), it is tempting to speculate that CD3\(\epsilon\gamma\) is the cave-associated CD3\(\epsilon\) heterodimer. Limited glycosylation of CD3\(\gamma\) relative to CD3\(\delta\) allows for ready modeled docking of the CD3\(\delta\) but not CD3\(\epsilon\) heterodimer at this cavity while still accommodating H57 mAb binding (unpublished results). Three additional facts also support the presumptive CD3\(\epsilon\gamma\) localization. First, it is known from chain association studies that CD3\(\epsilon\) and TCR\(\beta\) pairs with TCR\(\alpha\) whereas the CD3\(\epsilon\gamma\) heterodimer binds to TCR\(\beta\) (53). Second, these experiments show that CD3\(\epsilon\) and TCR\(\beta\) interact via their respective ectodomains whereas the associations of the CD3\(\gamma\), \(\delta\), and \(\zeta\) components with TCR\(\alpha\) and \(\beta\) are mediated via their transmembrane regions. Third, chemical cross-linking experiments in man and mouse imply proximity of TCR\(\beta\) and CD3\(\gamma\) (18, 54). The above results notwithstanding, verification of these predictions still requires definitive structural elucidation.

The inability of the C\(\beta\) FG loop deletion to influence the function of the pre-TCR is consistent with recent transgenic experiments in the p\(\alpha\) transgenic background: p\(\alpha\) constructs lacking both the ectodomain and much of the cytoplasmic tail still support pre-TCR function (55). These data imply that p\(\alpha\) stabilizes the pre-TCR predominantly via transmembrane interactions, with CD3\(\epsilon\) heterodimer association not singularly dependent on the cave for pre-TCR function. Further support for this notion comes from three other observations: (a) mice transgenic for a TCR\(\beta\) chain lacking the VB domain support DN to DP transition (56); (b) p\(\alpha\) and TCR\(\beta\) chains lacking their respective Ig-like domains still facilitate thymic differentiation in RAG-deficient mice (57); and (c) mutational analysis of the two tyrosines in the TCR\(\beta\) transmembrane region show that even conservative tyrosine to phenylalanine changes disrupt thymocyte survival, proliferation, and differentiation (58), further underscoring the importance of the transmembrane segment for pre-TCR function. Alterations within the TCR ectodomains are less well tolerated with regard to \(\alpha\beta\)TCR function, significantly impinging on TCR activity. For example, C\(\beta\) FG loop deletion reduces the efficiency of negative selection in N15αβ,\(\beta\Delta\)FG RAG-2\(^{-/-}\) mice. Mutations of the TCR\(\alpha\) chain connecting peptide profoundly affect positive selection. The latter occurs through attenuation of ERK activation and a reduction of activated Ick, Zap-70, phospho-CD3\(\epsilon\), and phospho-LAT within lipid rafts (59). A similar phenotype with equivalent biochemical signaling aberrations is observed in CD3\(\epsilon\gamma\) mice (60). If CD3\(\epsilon\gamma\) maps to the TCR\(\beta\) side and CD3\(\epsilon\delta\) to the TCR\(\alpha\) side of the \(\alpha\beta\) TCR complex, these findings can be rationalized.

In N15αβ,\(\beta\Delta\)FG RAG-2\(^{-/-}\) H-2\(^b\) mice, the level of N15 TCR as judged by both MR9.4 (anti-V\(\beta\)B) or R53 (anti-V\(\beta\)B clonotype) is virtually identical to that found on T cells of N15αβ RAG-2\(^{-/-}\) H-2\(^b\) mice. However, in the former case, ~50% of TCRs are wild-type and 50% incorporate the mutant \(\beta\Delta\)FG subunit. Yet mature CD8\(^+\) SP T cell function is significantly reduced in N15αβ,\(\beta\Delta\)FG RAG-2\(^{-/-}\) mice. Thus, despite half of the TCRs being wild-type in structure, functional activation of these T cells vis-a-vis cytokine production or proliferation is dramatically reduced. In contrast, tetracycline regulatable TCR transgene expression shows that cells expressing as few as 1/20 of the normal levels of TCRs preserve their functional activity (61). Thus, paradoxically, the presence of TCRs with the \(\beta\Delta\)FG subunit is more detrimental to TCR-triggered activation than the complete absence of wild-type TCRs. Although the basis for this disparity remains to be elucidated, N15αβ,\(\beta\Delta\)FG TCRs may disregulate wt TCRs by activating inhibitory pathways (phosphatases or others) in the absence of productive stimulation.

These data indicate an important functional role for the C\(\beta\) FG loop in both thymic negative selection and mature T cell activation. T lineage activities requiring threshold stimulation to achieve a stringent set point. On the other hand, positive selection is only modestly altered. In view of evidence that positive selection requirements vis-a-vis pMHC interaction are less arduous with regard to affinity and avidity than those for negative selection (47, 48), this result is perhaps not surprising. Thus, attendant reduction in signaling strength through TCRs incorporating mutant \(\beta\Delta\)FG subunits may still be sufficient to achieve the more
“relaxed” set point threshold for successful positive selection. Apparently, structural features of the αβ TCR have evolved to facilitate the “multi-tasking” requirements of TCR signaling function during development and mature T cell function. In sum, TCRβ-linked transduction events are more critically involved in negative selection while it appears that TCRα-linked transduction processes are key for positive selection.

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