Expression of fully assembled TCR–CD3 complex on double positive thymocytes: synergistic role for the PRS and ER retention motifs in the intra-cytoplasmic tail of CD3ε

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Abstract

TCR expression on double-positive (DP) thymocytes is a prerequisite for thymic selection that results in the generation of mature CD4+ and CD8+ single-positive T cells. TCR is expressed at very low level on preselection DP thymocytes and is dramatically up-regulated on positively selected thymocytes. However, mechanism governing TCR expression on developing thymocytes is not understood. In the present report, we demonstrate that the intra-cytoplasmic (IC) domain of CD3ε plays a critical role in regulating TCR expression on DP thymocytes. We provide genetic and biochemical evidence to show that the CD3ε IC domain mutations result in elevated expression of fully assembled TCR on DP thymocytes. We also demonstrate that TCR up-regulation on DP thymocytes in these transgenic mice occurs in a ligand-independent manner. Further, we show that the proline-rich sequence and endoplasmic reticulum (ER) retention motifs in the IC domain of CD3ε play synergistic role in regulating TCR surface expression on DP thymocytes.

Introduction

Development of αβ T cells in the thymus is a complex process that culminates in the generation of functionally mature T cells. The three stages of αβ T-cell development as defined by expression of CD4 and CD8 co-receptor molecules are: CD4−CD8− [double negative (DN)], CD4+CD8− [double positive (DP)] and CD4−CD8+ or CD4+CD8+ [single positive (SP)]. While the DN to DP transition is mediated by pre-TCR, the DP to SP transition is mediated by TCR (1–4). Pre-TCR expresses pTα/TCRβ heterodimer, whereas TCRα chain replaces the pTα chain in TCR. Both the types of receptors are associated with invariant CD3γ, δ, ε and ζ chains.

αβTCR/CD3 expression on DP thymocytes is essential for thymic selection. Preselection DP thymocytes express ~10- to 10-fold lower TCR compared with SP thymocytes or mature T cells indicating dynamic regulation of TCR expression during thymocyte development (5). TCR on DP thymocytes interacts with self-peptide MHC (pMHC) resulting in thymic selection and differentiation into CD4 and CD8 SP thymocytes, which exit the thymus and populate peripheral lymphoid organs. The avidity of TCR–pMHC interactions, which depends on the affinity and the expression levels of TCR and/or ligand, significantly influences thymic selection (6–10). Low TCR levels on preselection DP thymocytes have been suggested to compensate for high sensitivity of these cells, compared with mature T cells, for low-affinity ligands (11–14).

Several mechanisms have been proposed to explain regulation of TCR expression on developing thymocytes. Post-transcriptional modification of receptor components has been proposed as one of the mechanisms for regulating TCR levels on preselection DP thymocytes (15). Biochemical and genetic experiments have shown that MHC class II–CD4 interactions, which results in activation of Lck, a Src family...
tyrosine kinase, play an important role in maintaining low TCR levels on DP thymocytes (16). Accordingly, MHC class II or Lck deficiency results in elevated TCR expression on DP thymocytes (17, 18). Lck-mediated phosphorylation of the CD3 chains has been suggested to result in endocytosis of the receptor. The endocytosed receptor interacts with Src-like adapter protein (SLAP) and c-Cbl complex leading to ubiquitination and degradation of CD3ɛ chain resulting in lower TCR expression on DP thymocytes (19).

The CD3 chains play essential roles in ordered intracellular assembly and transport of TCR–CD3 complex to cell surface, receptor internalization (ligand dependent and ligand independent) and differential signal transduction. Cell transfection studies have identified di-leucine-based endocytosis motif in the intra-cytoplasmic (IC) domain of CD3γ and CD3δ (20–22). Similarly, a role for immunoreceptor tyrosine-based activation motif (ITAM) in receptor endocytosis has been suggested for the CD3 chains (20, 23, 24). However, mutating the ITAM of individual CD3 chain does not appear to affect TCR expression on developing thymocytes (25–30). Thus, it remains unclear how TCR expression is regulated on DP thymocytes. Recently, we and others showed that the proline-rich sequence (PRS) motif in the IC domain of CD3ɛ plays a role in regulating TCR expression on DP thymocytes ([31, 32] and the present report). Mutating the CD3ɛ: PRS motif was shown to result in defective degradation of internalized CD3ɛ chain leading to increased TCR expression on preselection DP thymocytes (32).

Apart from the endocytosis, the endoplasmic reticulum (ER) retention and degradation of partially assembled or misfolded TCR–CD3 complex play a role in regulating TCR expression (reviewed in refs 5, 20). Biochemical studies have shown that CD3γ and CD3δ heterodimers preferentially associate with TCRβ and TCRα, respectively. The final step in the receptor assembly is association of the CD3ɛɛ homodimer with the TCRββCD3γδα partial complex and transport of the fully assembled TCR–CD3 complex to cell surface (33–36). Incorporation of the CD3ɛɛ homodimer has been suggested to mask the ER retention/degradation and/or di-leucine motifs present in the CD3γ, δ and ε chains, usually in their IC domain (5, 20, 37, 38). This ensures exit of fully assembled receptor from the ER/Golgi and transport to the cell surface. In agreement with this, lack of CD3ɛ or its inability to associate with partially assembled TCR complex for instance, in CD3ɛɛ mice, results in lower TCR surface expression (39–42). However, cell transfection studies showed that mutating the ER retention motif in the CD3ɛ IC domain results in expression of CD3ɛɛ and CD3ɛɛ-deficient TCRββCD3γɛ partial complexes suggesting dominant role for this motif in TCR expression (43).

Apart from dispensable nature of the tyrosine-based motif of the CD3 chain in modulating TCR expression, role of the ER retention motif, particularly of CD3ɛ, in regulating TCR expression on developing thymocytes has not been studied. As well, the effect of mutating the ER retention and PRS motifs of CD3ɛ on TCR expression on developing thymocytes has not been tested. In the present investigation, we provide evidence to show that the IC domain of CD3ɛ plays critical role in regulating surface expression of fully assembled TCR on DP thymocytes in a ligand-independent manner. We also demonstrate that the PRS and ER retention motifs of CD3ɛ play synergistic role in regulating TCR expression on DP thymocytes.

**Methods**

**Mice**

CD3ɛɛɛ (44) and CD3ɛɛɛɛ (42) mice have been described previously. TCRɛɛɛɛɛɛ and MHCɛɛɛɛɛɛ mice were obtained from The Jackson Laboratory and Taconic, respectively. The various transgenes were generated by amplifying cDNA-encoding CD3ɛ chain and cloned into human CD2 expression cassette that permits transgenic expression throughout T-cell ontogeny (45). At least two independent founder lines were established for each transgenic construct. Analyses of all the transgenic mice (except the ER retention motif mutated) described in the present report are described in detail elsewhere (31). C57Bl/6 mice were used as controls. All mice were bred and maintained in a specific pathogen-free animal facility at the Institut de Recherches Cliniques de Montreal. Animal care was in accordance with the Canadian Committee on Animal Care guidelines.

**Antibodies and flow cytometry**

Fluorescently labeled antibodies to CD3, CD4, CD8, TCR, CD69, CD5, CD25, CD44 and AnnexinV were obtained from Pharmingen or EBiocience. Anti-CD3ɛ (2C11) antibody was purified from hybridoma supernatant using protein A Sepharose column. Anti-CD3ɛ mAb (clone H146) and polyclonal rabbit serum (p551) were kind gift of Andre Veillette, Al Singer and David West. Anti-CD3ɛ antibody (clone 6B10) was purchased from Santa Cruz Biotech, Santa Cruz, CA, USA. Thymus and lymph nodes (LNs) were harvested from 6- to 8-week-old mice and single-cell suspension was prepared in RPMI containing 0.2% FCS (Invitrogen Inc). A total of 1–2 × 10^5 thymocytes or LN cells were stained with fluorescently labeled antibodies at 4°C for 15 min, washed in RPMI/FCS and analyzed by FACScalibre.

For intracellular staining, 1 × 10^6 thymocytes or LN cells were stained for surface receptor, washed, fixed in 2% PFA, permeabilized in 0.5% saponin in RPMI/FCS. Fixing and permeabilization steps were carried for 20 min in dark at room temperature. Samples were first incubated with hamster IgG and/or 2.4G2 culture supernatant followed by fluorescently labeled anti-CD3ɛ antibody (17A2) or anti-TCRβ antibody (H57).

**Protein analysis**

Fifty million thymocytes were lysed for 45 min on ice in Triton X-100 lysis buffer (1% Triton X-100, 50 mM Tris–HCl (pH7.6), 100 mM NaCl, EDTA, protease inhibitor) and centrifuged for 10 min in cold. Supernatant was precleared by incubating with protein A Sepharose beads. TCR complexes were immunoprecipitated with 2C11 antibody, washed twice in lysis buffer containing 0.2% Triton X-100, eluted in SDS sample buffer, resolved by SDS–PAGE and western blotted using anti-CD3ɛ antibody. For sequential immunoprecipitation of surface biotinylated receptor, 50 × 10^6 to 100 × 10^6 thymocytes were washed in HBSS containing 1 mM MgCl2.
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and 0.1 mM CaCl₂ (HBSS), suspended in 1 ml HBSS and cooled for 30 min on ice. Cooled thymocytes were mixed with 500–750 µg of sulfo-NHS-biotin (Pierce Chemical) for 30 min on ice. The biotinylation reaction was quenched by adding HBSS containing 25 mM lysine monochloride and washed two times in HBSS. More than 95% of cells were viable as determined by trypan blue exclusion. Cells were lysed for 45 min on ice in 1% digitonin lysis buffer and centrifuged for 10 min in cold. Supernatant was incubated with rabbit anti-mouse IgG-coupled protein A sepharose beads. The precleared lysates were incubated with 2 µg of mouse anti-human CD3ɛ mAb (clone 6B10; the antibody recognizes mouse and human CD3ɛ) followed by rabbit anti-mouse IgG-coupled protein A sepharose beads. The antibody incubation and immunoprecipitation steps were repeated two more times. The lysates were finally immunoprecipitated with 2C11-coupled protein A sepharose beads. The immunoprecipitated proteins were resolved by SDS-PAGE and western blotted with avidin-coupled HRP protein A. In some cases, digitonin lysates of surface biotinylated thymocytes were immunoprecipitated with anti-CD3ɛ antibody (H146) and western blotted (Fig. 3C).

Results

CD3ɛ: IC domain mutation up-regulates TCR expression on DP thymocytes

The IC domain of CD3ɛ contains, from the membrane proximal end, basic amino acid-rich sequence (BRS), PRS, ITAM and ER retention motif. To determine the role of these motifs, we generated several CD3ɛ transgenic mice. Characterization of various CD3ɛ transgenic mice is described elsewhere (31). We previously showed that the ε161 (expressing the BRS motif), the ε170 (expressing the BRS+PRS motifs; please note the function of PRS motif in this transgene is severely impaired; (46,47)) and the εPRS (mutated only in the PRS motif) transgenes restored DN to DP differentiation in CD3ɛ−/− mice, although thymic cellularity was rescued only partially (Fig. 1; please note similar data are provided in ref. 31). The frequency and absolute numbers of SP thymocytes and mature T cells in the spleen of three transgenic mice were quite comparable with wild-type or εFL transgenic (expressing full-length CD3ɛ) mice indicating normal thymic selection of endogenous TCR repertoire (Fig. 1 and data not shown). However, the three transgenes affected TCR levels on thymocytes in a development stage-specific manner. TCR expression on DP, but not DN, thymocytes was dramatically up-regulated in ε161 and ε170 transgenic mice. Thus, there was ~10-fold increase in the number of DP thymocytes with elevated TCR expression in ε161 and ε170 transgenic mice compared with wild-type mice (Fig. 1). Similarly, εPRS transgenic mice also showed an increase, albeit moderate, in the number of DP cells with increased TCR levels compared with wild-type control. There was ~3-fold more DP thymocytes with high TCR levels in εPRS mice compared with control mice. TCR levels were also increased on SP thymocytes and mature T cells from ε161 and ε170, but not εPRS, transgenic mice.

To confirm up-regulation of TCR on DP thymocytes, we introduced the ε170 transgene into CD3ɛ−/−/TCRα−/− mice. As shown in Fig. 2, the thymic phenotype of ε170CD3ɛ−/−/TCRα−/− mice is essentially the same as that of TCRα−/− mice, i.e., the presence of only the DN and DP thymocytes and the absence of thymocytes expressing increased amounts of TCR.

Data described above clearly show that mutating the IC domain of CD3ɛ: results in increased TCR expression on DP thymocytes. Since TCR complexes contain two chains of CD3ɛ, we wondered if the effect of mutant form of CD3ɛ: on TCR levels on DP thymocytes was dominant compared with the full-length protein. To this end, we introduced the εIC-del transgene into wild-type mice (middle panels in Fig. 3; and ref. 31). We previously showed that the εIC-del transgene is expressed at levels comparable to wild-type CD3ɛ and heterodimerizes efficiently with CD3γ and CD3δ and still fails to restore thymic development in CD3ɛ−/− mice (31). We also showed that the εIC-del transgene does not affect thymocyte development in wild-type mice (31) and thus, allows us to determine the effect of mutant CD3ɛ on TCR levels on DP thymocytes in the presence of wild-type CD3ɛ. To this end, we introduced the εIC-del transgene into wild-type mice and TCR level on developing thymocytes was analyzed. Wild-type mice expressing the εIC-del transgene showed a significant increase in TCR expression on DP thymocytes; there was ~5-fold increase in number of DP thymocytes with elevated TCR level. Similar to the εIC-del transgene, the ε161, ε170 or εPRS transgene also up-regulated TCR levels on DP thymocytes in wild-type mice (data not shown). These data suggest a dominant effect of the IC domain-mutated CD3ɛ on TCR expression on DP thymocytes in wild-type mice.

Although the IC domain-deleted CD3ɛ: up-regulated TCR levels on DP thymocytes in the presence of wild-type CD3ɛ: we wished to determine if the dominant effect was specifically due to the IC domain-mutated CD3ɛ: To test this notion, we introduced a chimeric CD3ɛ molecule expressing the IC domain of CD3γ instead of CD3ɛ into wild-type mice. We previously showed that the chimeric molecule, εγɛ, fails to rescue thymic differentiation in CD3ɛ-deficient mice. We also showed that the inability of εγɛ to rescue thymic differentiation in CD3ɛ-deficient mice was not due to lower expression and/or its ability to heterodimerize with CD3ɛ indicating that the IC domain of CD3ɛ is specifically required for thymocyte development (31). To assess the effect of εγɛ transgene on TCR expression on DP thymocytes, we introduced this transgene into wild-type mice. Surprisingly, the εγɛ transgene failed to up-regulate TCR levels on DP thymocytes (Fig. 3, bottom panels). Anti-CD3ɛ: immunoprecipitates followed by avidin blot of surface biotinylated thymocytes isolated from wild-type mice expressing εIC-del or εɛɛ transgene readily detected the truncated or chimeric protein indicating that the opposing effect of the two transgenes on TCR expression on DP thymocytes was not due to low expression or their inability to associate with the receptor complex (Fig. 3C). The transgene did not affect any aspect of thymocyte development in wild-type mice and accordingly, all the thymocyte subsets were detected in normal numbers (Fig. 3). Thus, the εIC-del and εɛɛ transgenes, although failed to rescue thymic differentiation in CD3ɛ-deficient mice, had opposing effect on TCR expression on DP thymocytes in wild-type mice. Taken together, these data suggest that mutating the IC domain of CD3ɛ: elevates TCR
levels on DP thymocytes in a dominant manner and that this effect does not appear to be due to specific mutations in the IC domain of CD3ε.

CD3ε IC domain mutation regulates expression of fully assembled TCR

Elevated TCR expression and up-regulation of CD69 on developing thymocytes are hallmarks of positive selection. However, an increase in TCR expression on DP thymocytes in ε161, ε170 and εPRS transgenic mice appeared not to be due to increased efficiency of thymic selection as the frequency of SP thymocytes in these mice was quite comparable to control animals. In agreement with this, the majority of DP thymocytes from the three transgenic mice (ε161, ε170 and εPRS) were CD69low indicating that these thymocytes were not selected (Fig. 4). CD5 expression, a negatively regulator of TCR signaling, is strongly correlated with TCR levels (48). Interestingly, there was only a small increase in CD5 expression on DP thymocytes from ε161 and ε170, but not εPRS, transgenic mice.

Since cell transfection studies showed that mutating the ER retention motif in the IC domain of CD3ε permits surface expression of CD3δ- and CD3ζ-deficient partial TCR complexes (43), we reasoned that the majority of TCR on DP thymocytes from the three transgenic mice (ε161, ε170 and εPRS) may lack either or both CD3 chains resulting in inefficient thymic selection. Although we previously showed that the transgene-encoded proteins efficiently heterodimerize with CD3c and CD3d, it was possible that elevated levels of incomplete TCR complexes lacking CD3ζ were expressed on DP thymocytes in these transgenic mice. To test this notion, we took a biochemical approach. Since incorporation of the CD3ζζ homodimer into TCR complex is the final step in receptor assembly, we reasoned that if the majority of TCR complexes on DP thymocytes in CD3ε transgenic mice is complete then the amount of CD3ζ chain associated with TCR–CD3 complex should be more in the transgenic than wild-type thymocytes. To test this notion, we immunoprecipitated thymocytes from wild-type and CD3ε transgenic mice with 2C11 antibody and western blotted with anti-CD3ζ antibody. As shown in Fig. 5(A and B), ~2- to 3-fold more CD3ζ could

Fig. 1. Effect of CD3ε IC domain mutations on TCR expression on thymocytes. (A) Schematic presentation of various CD3ε transgenic constructs is shown. Single letter amino acid sequence for the IC region is shown, and various motifs are depicted. The ε161 transgene expresses only the BRS motif, the ε170 transgene expresses the BRS and PRS (functionally weak) motifs, whereas the εPRS transgene has all five proline residues in the PRS motif mutated to alanine. (B) Thymocytes and (C) LN cells from wild-type, ε161/C0/C0, ε170/C0/C0 and εPRS/C0/C0 mice were analyzed by flow cytometry using antibodies to CD4, CD8 and TCRβ. The percentages of various thymocyte subsets and LN T cells are shown in the respective quadrants. The numbers in the boxes in the TCRβ histograms of the gated DP thymocytes shows percentages of cells with elevated TCR. Mean fluorescent intensity of TCRβ staining is shown for the gated CD4+ and CD8+ SP T cells. (D) Shows percent of TCRhigh DP thymocytes in mice of the indicated genotype in bar graph. Data are representative of at least four independent experiments. Please note similar data are reported in ref. 31.
strongly suggest that the CD3ε IC domain mutation up-regulates expression of the fully assembled TCR on DP thymocytes.

**Ligand-independent increase in TCR expression on DP thymocytes in CD3ε transgenic mice**

Since TCR–pMHC interaction results in up-regulation of TCR levels on positively selected thymocytes, we wondered if elevated TCR expression on DP thymocytes in CD3ε transgenic mice required this interaction. Although CD69 staining data suggest that the majority of DP thymocytes in CD3ε transgenic mice is not selected, it remained possible that these cells required low avidity receptor–ligand engagement for up-regulating TCR levels on DP thymocytes but fail to undergo thymic selection. A moderate increase in CD5high DP thymocytes in ε161 and ε170 transgenic mice points to such a possibility. To this end, we backcrossed ε170CD3ε−/− and MHC−/− (mice deficient in MHC class II−/− and B2M−/−) mice to obtain ε170CD3ε−/−/MHC−/− mice and thymocytes from these mice were analyzed. Figure 6 shows analyses of thymocytes from mice of the indicated genotype. Indeed, the TCR surface expression on DP thymocytes was up-regulated to the same extent in ε170CD3ε−/−/MHC−/− and ε170CD3ε−/− transgenic mice (Fig. 6). These data strongly suggest that mutating the CD3ε IC domain motifs allows surface expression of the fully assembled TCR complexes on DP thymocytes independent of receptor–ligand engagement.

**Synergistic role for the PRS and ER retention motifs of CD3ε in regulating TCR levels on DP thymocytes**

Thymocytes from ε161 and ε170 transgenic mice showed a dramatic increase in the number of DP thymocytes with elevated TCR levels (~10-fold), whereas those from εPRS transgenic mice showed only a modest increase (~3-fold). The ε161 and ε170 transgenes lack the ER retention and PRS motifs, whereas the εPRS transgene is mutated in the PRS motif only. Thus, it was possible that differential effects of these mutations on TCR expression on DP thymocytes were due to dominant role of the ER retention motif compared with the PRS motif. Alternately, elevated TCR expression on DP thymocytes from CD3ε transgenic mice could be additive or synergistic effects of mutating both the motifs in the ε161 and ε170 transgenes. To address this issue, we generated a new transgenic line in which the last five amino acids in the IC domain of CD3ε were deleted. Thus, this transgene, εAER, expresses all the motifs (BRS, PRS and ITAM) except the ER retention motif (transgene εAER, Fig. 7A).

Analysis of εAER transgenic mice confirmed several conclusions deduced from the analyses of various CD3ε transgenic mice described previously (31). Similar to ε161, ε170 and εPRS transgenic mice, εAER mice also showed normal DN to DP transition and the presence of mature T cells (Fig. 7B and C) even though the transgene was expressed at slightly lower level than wild-type control as judged by the intracellular anti-CD3 staining of thymocytes (Fig. 7E). However, εAER transgenic mice showed significantly more efficient DN3 to DN4 differentiation compared with ε161, ε170 or εPRS mice ([31, 32]; Fig. 7D and data not shown). Second, thymic cellularity was completely restored...
in eAER mice indicating that the PRS motif is important for efficient DN3 to DN4 differentiation as well as proliferation of DN thymocytes. Third, the number of DP thymocytes expressing increased level of TCR, while increased by ~3-fold compared with wild-type control, was significantly less when compared with those from e161 or e170 transgenic mice. Comparing DP thymocytes from ePRS and eAER transgenic mice to those from e161 and e170 transgenic mice strongly suggest synergistic effects of mutating the PRS and ER retention motifs of CD3ε on TCR levels on DP thymocytes. Finally, the eAER transgene up-regulated TCR levels on DP thymocytes in wild-type mice as well. Taken together, these data provide strong genetic and phenotypic evidences for synergistic roles for the CD3ε PRS and ER retention motifs in regulating TCR expression on DP thymocytes.

**Discussion**

In the present investigation, we have attempted to elucidate roles of the CD3ε IC domain motifs in regulating TCR expression on developing thymocytes. Altered TCR expression on DP thymocytes significantly modulates thymic selection (5, 6, 9, 10). Exit from the ER and transport of the fully assembled receptor to the cell surface and receptor internalization are two mechanisms of regulating TCR surface expression. CD3 chains play an essential role in TCR assembly, transport and receptor internalization. Defect in assembly and/or transport due to lack of or mutating one of the components of TCR complex results in the ER retention and degradation of partial TCR complexes. The function of CD3 chains in the endocytosis or ER retention of the receptor is predominantly mediated by their IC domain, although the extracellular domain has also been reported to contain the ER retention sequence (20, 43).

We previously showed that the CD3ε IC domain plays essential and multiple roles in pre-TCR-mediated differentiation and proliferation of DN thymocytes (31). We showed that the presence of BRS motif in the IC domain of CD3ε is sufficient for mediating DN to DP differentiation, whereas the PRS motif plays an important role in DN3 to DN4 differentiation and proliferation. Thymic phenotype of e^{161}CD3ε170/− mice described in the present report supports this conclusion. However, mutating the IC domain of CD3ε does not alter pre-TCR expression suggesting that the ER retention/ endocytosis of pre-TCR is stringently regulated and cannot be overcome by mutating the IC domain of individual CD3 chains or pTα (49). In contrast, TCR expression on DP thymocytes is significantly affected by mutations in the IC domain of CD3ε but not CD3γ or CD3δ (S.L. and V.P.D., unpublished observation).

In the present report, we show that mutating all but the BRS motifs in the IC domain of CD3ε (e^{161}) dramatically up-regulates TCR expression on DP thymocytes. A recent report showed that the CD3ε BRS motif, which contains positively charged residues, interacts with membrane lipid (50). Whether this potential interaction plays any role in pre-TCR-mediated DN to DP transition or TCR expression is under investigation. Similar to the e^{161} transgene, the e^{170} transgene also up-regulated TCR on DP thymocytes. The e^{170} transgene was generated by mutating the tyrosine170 to a...
stop codon Fig. 1 and (31). This tyrosine is suggested to be a part of the PRS motif (46,47) and mutating this tyrosine affects the PRS-mediated CD3ε–Nck interaction (31). Thus, the PRS, ITAM and ER retention motifs are mutated in the e170 transgene. In contrast to dramatic effect of mutating the PRS and ER retention motifs on TCR expression on DP thy- mocytes, mutating only the PRS (ePRS transgene) or the ER retention motif (eD ER transgene) showed only a moderate in- crease in the number of DP thymocytes with elevated TCR levels. It is not clear why mutating the PRS or ER retention motif affects TCR expression only on a fraction of thymo- cytes. In the case of ePRS transgenic thymocytes, this may reflect defective β-selection (31, 32), whereas in the case of eGER, it may be due to rapid endocytosis of high-affinity TCRs (51).

While our data suggest that the IC domain-deleted CD3ε elevates TCR expression on DP thymocytes even in the presence of wild-type CD3ε suggesting a dominant effect of the mutant protein, this effect does not appear to be due to the lack of CD3ε IC domain as the chimeric eγγ molecule expressing the IC domain of CD3γ instead of CD3ε did not alter TCR expression on DP thymocytes. In other words, the chimeric eγγ behaves as wild-type CD3ε in regulating TCR expression on DP thymocytes. We propose that the IC domain of CD3γ stabilizes the quaternary structure of the receptor and/or potential interactions between receptor and ER chaperone and that the association of CD3f masks all the ER retention motifs. That the receptor structure, particu- larly the IC domain structure, may be altered, albeit indirectly, by biochemical and phenotypic data. These data show that association of the CD3ε chain is not sufficient in masking the ER retention motifs of TCR–CD3 complex in CD3ε transgenic mice (except eγγ). Alternately, the presence of the IC domain of CD3γ in eγγ transgene, which contains the ER retention and di-leucine-based internalization motifs, may restore proper ER retention/degradation of partial TCR complexes as well as endocytosis/degradation of surface TCR complexes on DP thymocytes in eγγ transgenic mice (in wild-type background).

Although genetic and phenotypic data strongly suggest synergistic effects of mutating the CD3ε ER retention and PRS motifs on TCR expression on DP thymocytes, it is possible that the ITAM of CD3ε may also contribute to TCR expression on DP thymocytes. However, this seems unlikely...
as mutating the ITAM has not been reported to increase TCR levels on DP thymocytes (25) or affect the TCR endocytosis in any significant manner (23). Further, recent studies show that mutating the CD3e PRS motif affects ITAM tyrosine phosphorylation (47, 52). Based on these data, mutating the PRS motif could be equated to mutating the ITAM tyrosine. CD3e transgene mutated in the PRS and ER retention motifs or the ITAM and ER retention motifs will confirm this notion.

What may be the purpose of having two independent mechanisms of regulating TCR expression during thymocyte development is not clear. We propose that the ER retention motif of CD3e ensures exit of the fully assembled receptor, whereas the PRS motif plays a role in degradation of internalized receptor and thymic selection ([32]; the PRS motif also plays an independent role in mediating the DN3 to DN4 transition and proliferation of DN thymocytes). Expression of the partially assembled receptor in the absence of one of the receptor components, for example, CD3f chain, may result in the generation of autoreactive T cells (53, 54). What role receptor endocytosis and/or ER retention plays in thymic selection and/or mature T cell activation is under investigation. It is interesting to note that increased TCR expression on DP thymocytes from the three CD3e transgenic mice (e161, e170 and ePRS) does not result in enhanced thymic selection of endogenously rearranged TCR. This is likely due to weakened signal transduction via TCR–CD3 complex lacking the PRS and/or ITAM motif (26, 32). In agreement with this, preliminary data show that thymic selection of HY-TCR in e161 transgenic mice is severely impaired. It will be particularly interesting to determine how up-regulation of TCR on DP thymocytes affects thymic selection in mice.
expressing all the CD3e motifs except the ER retention motif. While the CD3e IC domain mutations affect TCR expression on DP thymocytes dramatically, they seem to affect TCR levels on mature T cells only moderately. Preliminary data show a marginal reduction in in vitro proliferation of mature T cells from the CD3e transgenic mice indicating dispensable nature of the PRS and/or the ER retention motif in mature T cell function and is in agreement with a published report (32).

While our data show that the CD3e IC domain plays critical role in regulating TCR expression at thymic selection stage, we also show that TCR up-regulation on DP thymocytes in CD3e transgenic mice does not require TCR–pMHC interactions. What may then be the biological purpose for controlling TCR surface expression in a ligand-dependent and developmental stage-specific manner? One reason for regulating TCR levels on preselection DP thymocytes may be to compensate for sensitivity of these cells for pMHC ligand (11–14). A recent report showed the generation of mature αβ T cells in mice deficient in MHC and co-receptor molecules (B2m−/−II−/−CD4−/−CD8−/−; quad deficient; ref. 55). These T cells up-regulated surface TCR but only after being selected by non-MHC ligand and were shown to be autoreactive. We propose that CD3e-mediated regulation of TCR expression only at the DP stage may prevent the development of self-reactive MHC-independent T cells that may mature in the absence of CD4/CD8 co-receptor for instance, at the DN stage of thymocyte differentiation. Whether CD3e regulates surface expression of endogenously expressed TCR at the DN stage remains to be investigated. In conclusion, we have shown that the IC domain of CD3e is critical for regulating TCR expression at the DP stage and that the PRS and ER retention motifs play synergistic role in it. It will be interesting to determine if CD3e IC domain mutation affects the stability of other components for instance, TCRα chain, of receptor complex. The outcome of these experiments will provide a better understanding of the mechanisms of TCR expression and thymocyte differentiation and role of CD3e chain in it.

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Abbreviations
BRS basic amino acid-rich sequence
DN double negative
DP double positive
ER endoplasmic reticulum
Role of CD3δ: intra-cytoplasmic tail in TCR expression on DP thymocytes

References


