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Distinctive CD3 Heterodimeric Ectodomain Topologies Maximize Antigen-Triggered Activation of αβ T Cell Receptors

Sun Taek Kim,*† Maki Touma,*† Koh Takeuchi,‡ Zhen-Yu J. Sun,§ Vibhuti P. Dave,∥ Dietmar J. Kappes,∥ Gerhard Wagner,‡ and Ellis L. Reinherz*‡

The αβ TCR has recently been suggested to function as an anisotropic mechanosensor during immune surveillance, converting mechanical energy into a biochemical signal upon specific peptide/MHC ligation of the αβ clonotype. The heterodimeric CD3εγ and CD3ζδ subunits, each composed of two Ig-like ectodomains, form unique side-to-side hydrophobic interfaces involving their paired G-strands, rigid connectors to their respective transmembrane segments. Those dimers are laterally disposed relative to the αβ heterodimer within the TCR complex. In this paper, using structure-guided mutational analysis, we investigate the functional consequences of a striking asymmetry in CD3γ and CD3ζ G-strand geometries impacting ectodomain shape. The uniquely kinked conformation of the CD3γ G-strand is crucial for maximizing Ag-triggered TCR activation and surface TCR assembly/expression, offering a geometry to accommodate juxtaposition of CD3γ and TCR β ectodomains and foster quaternary change that cannot be replaced by the isologous CD3ζ subunit’s extracellular region. TCRβ and CD3ζ subunit protein sequence analyses among Gnathostomata species show that the Cβ FG loop and CD3ζ subunit coevolved, consistent with this notion. Furthermore, restoration of T cell activation and development in CD3γCCR−/− mouse T lineage cells by interspecies replacement can be rationalized from structural insights on the topology of chimeric mouse/human CD3ζδ dimers. Most importantly, our findings imply that CD3γ and CD3ζ evolved from a common precursor gene to optimize peptide/MHC-triggered αβ TCR activation. The Journal of Immunology, 2010, 185: 2951–2959.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: 17A2-A647, 17A2-Alexa Fluor 647; 2C11-F, 2C11-FITC; Cyt, cyttoplasmic; DN, double negative; FTOC, fetal thymic organ culture; hCD3, human CD3; LN, lymph node; mCD3, murine CD3; MFI, mean fluorescence intensity; mut, mutant; NMR, nuclear magnetic resonance; pMHC, peptide bound to MHC; g, transgenic; TM, transmembrane; wt, wild-type.

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becomes stimulatory only subsequent to application of an external tangential (but not normal) force of ~50 pN introduced via optical tweezers. Specific pHMC, but not irrelevant pHMC, activates a T cell upon application of a similar force. During immune surveillance, when specific TCR-pHMC ligation occurs, an extracellular mechanical torque may be applied to the TCR prior to “stop movement” of the T cell. In this process, the pHMC on the APC functions as a gaff or hook to pull on the ligated TCR on the opposing T cell. Collectively, these findings suggest that the TCR is an anisotropic (i.e., directional) mechanosensor.

We reasoned that detailed analysis of the distinct topology of CD3 heterodimers would provide further insight into the basis of signal transduction involving the ectodomain components of the TCR mechanosensor. In addition, the rationale for differences in CD3 subunit requirements for various T lineage populations and developmental stages might emerge (19–28). In this study we have focused on differential G-strand geometry in CD3γ and CD3δ ectodomain fragments observed in human and other mammalian species. These features make the topology of CD3γ and of CD3δβ distinct from one another and, in turn, mandate a defined quaternary organization for the TCR. The findings suggest that despite the loose confedersy of dimeric ectodomains constituting the TCR complex, dynamic complementarity of structural elements maximizes sensitivity in the relay of quaternary change essential for signal transduction.

Materials and Methods

Abs and flow cytometric analysis

The following fluorochrome-labeled mAbs were used as surface receptor analysis by flow cytometry: FITC–anti-CD3 (2C11), Alexa Fluor 488–anti-CD3 (17A2), Pacific Blue-CD4 (H129.19), Pacific Orange anti-CD8α (53-6.7), PE–anti-γ/δ (MR9.4), and FITC-conjugated anti-TCR ε (H57) (BD Pharmingen, San Diego, CA). For flow cytometry, single-cell suspensions of thymocytes or lymph node (LN) cells were prepared at 5 × 10^6 cells/ml in PBS containing 2% FCS and 0.05% NaN3. Those cells were suspended T cells were cultured for 3 d with 50 μg/ml human rIL-2 until used for the assay. To test the TCR signaling of transduced N15TcrgCD3γ−/− T cells, cells were washed and stimulated with VS8 peptide-loaded irradiated splenocytes from C57BL/6 mice or with plate-coated 2C11 (5 μg/ml) for 4 h in media containing GolgiPlug reagent (BD Pharmingen). Then, cells were stained for surface CD8 and for intracellular IFN-γ, using Fix/Perm solution (BD Pharmingen).

Fetal thymic organ culture with retroviral transduction

For retroviral transduction with CD3γwt and mutant constructs, fetal thymus were removed from day 14.5 CD3γ−/− fetuses (observation of vaginal plug is day 0.5), and thymocytes at 100,000 cells/well (volume of 10 μl) were treated with 1.35 mM 2–deoxyguanosine (Sigma-Aldrich, St. Louis, MO) in transwell inserts (Costar, Cambridge, MA) for 5 d before use to remove hematopoietic cells, but not epithelial tissue capable of allowing the differentiation of T cell precursors. After 2 d of hanging culture, cells were transferred to ATTP 0.8 mM filters (Millipore, Billerica, MA) on Gelfoam (Pharmacia & Upjohn Company, Kalamazoo, MI). After 7 d, thymocytes were counted and analyzed by FACS. To determine transduction efficiency, 50,000 of the transduced cells were used for the FACS analysis of GFP expression.

Results

Structural comparison between CD3εγ and CD3δβ heterodimeric ectodomains

Solution structures of the murine CD3 (mCD3εγ) heterodimer and a chimeric CD3δβ heterodimer (mCD3ε and sheep CD3δ) reveal that both CD3ε domains adopt a virtually identical conformation with a root-mean-square deviation <1.45 Å (8, 9). Each heterodimer is composed of two Ig-like ectodomains with a hydrophobic interface brought together by hydrogen bond-paired terminal G-strands forming conjoint β-sheets. Unlike CD3ε, the CD3γ and CD3δ ectodomains within these heterodimers are more divergent from one another. In particular, structural comparison between CD3γ and CD3δ ectodomains shows that there is differential G-strand geometry resulting in a pronounced cleft between the two CD3 ectodomains in CD3δγ that is partially occluded in CD3δβ (Fig. 1A). The difference in G-strand disposition likely results from two factors. First, fewer hydrogen bonds are formed between the two G-strands in CD3δγ relative to those in CD3δβ, at least in part owing to an amino acid sequence of CD3γ that does not support the optimal packing between CD3ε and CD3γ at the N terminus (or top) of the G–G interaction surface. Second, relative to CD3γ, the CD3δ βC loop is five residues shorter, containing only four amino acids (Fig. 1B). The side view of the heterodimers in Fig. 1A (right panel) highlights the difference in CD3γ versus CD3δ FG and BC loops. The longer BC loop and the presence of the C′-strand force the entire GFCC′ face of CD3γ, along with the FG loop, to bow away from the heterodimeric interface, whereas the shorter BC loop in CD3δβ must cut across the two β-sheets (ABED and GFC′C′ faces), thereby

Retroviral transduction and peptide stimulation

For retroviral transduction, we used the pLZRS-ires-EGFP vector encoding the enhanced GFP downstream of an internal ribosome entry site (31). The virus supernatant was prepared as described previously (17, 32, 33). For retroviral transduction with CD3γwt and mutant constructs, total LN cells from N15TcrgCD3γ−/− mice were stimulated with 4 μg/ml concanavalin A for 2 d, and T cells were purified by removing I-Aβ+ positive cells using anti-I-Aβ and magnetic beads. N15TcrgCD3γ−/− T cells were placed in a 24-well plate at 10^5 cells/well (volume of 300 μl) in complete RPMI 1640 medium, and 300 μl viral supernatant containing 20 μg/ml Lipofectamin (Life Technologies-BRL, Carlsbad, CA) was added to each well and the plate centrifuged at 2000 rpm for 1 h at room temperature. Transduced T cells were cultured for 3 d with 50 μg/ml human rIL-2, T cells were washed and stimulated with VS8 peptide-loaded irradiated splenocytes from C57BL/6 mice or with plate-coated 2C11 (5 μg/ml) for 4 h in media containing GolgiPlug reagent (BD Pharmingen). Then, cells were stained for surface CD8 and for intracellular IFN-γ, using Fix/Perm solution (BD Pharmingen).

Mutagenesis of murine CD3γ

The cDNA encoding murine CD3γ was subcloned into the pcU18 for mutation and sequencing. To generate the CD3γΔ6M mutant in which the six amino acids in the G-strand of CD3γ (ETSNPL) were replaced with those of CD3δ (KVVSSV), we used the QuickChange Site-Directed Mutagenesis System (Stratagene, La Jolla, CA). CD3γΔ6M or CD3δγΔ6M mutant was constructed by replacing the whole ectodomain denoted in Fig. 1 by recombinant PCR methods. CD3γ wild-type (wt) or CD3γ mut- tant constructs all contained a C-terminal FLAG epitope (DYKDDDDK) for quantitation of protein expression. All generated constructs were confirmed by DNA sequencing.

CD3γ AND CD3δ G-STRAND GEOMETRIES
The preferential interaction of 17A2 with CD3ε cross-saturation analyses (15) showed that 17A2 contacts both CD3γ and CD3δ. Previous NMR binding experiments involving chemical shift and used tg mice generated on the CD3ε orthologs (34, 35). Note that the top of the hCD3ε FG loop (Gly52–Lys57) is missing in the existing crystal structure coordinates, however, most likely owing to the inherent flexibility in this region of the human ortholog.

Differential G-strand geometries of CD3γ and CD3δ are recognized by the 17A2 anti-mCD3ε mAb

Previous NMR binding experiments involving chemical shift and cross-saturation analyses (15) showed that 17A2 contacts both CD3ε and CD3γ domains, providing a structural explanation for preferential interaction of 17A2 with CD3ε on the T cell surface. To investigate regions of CD3γ critical for native 17A2 binding, we used tg mice generated on the CD3δ−/− background in which one of four segments of mCD3ε was replaced with a corresponding segment from mCD3γ (mutant [mut] A–D), as shown in Fig. 1B, and introduced into the mouse germline via transgenesis. The flow cytometric analysis in Fig. 2A reveals that both CD4 and CD8 T cells from CD3δ−/− mice express low levels of residual 2C11-FITC (2C11-F) reactivity (mean fluorescence intensity [MFI] = 127 and 119, respectively; red curves), consistent with low-level CD3εγ surface expression in the absence of CD3εδ (24). This 2C11-F staining is completely blocked by unlabeled 17A2 (MFI = 10–14; shaded blue curves). In contrast, the introduction of wtCD3δ on the CD3δ−/− background restores 2C11-F reactivity (MFI = 1362–2060), and this binding is inhibited only by ~50% upon preincubation with unlabeled 17A2 (see also schematic in Fig. 2A, right panel). Similar patterns of reactivity and partial blockade of 2C11-F staining by unlabeled 17A2 preincubation are seen with introduction of mutA, mutB, and mutC CD3δε transgenes. However, mutD, involving substitution of the G-strand and a portion of the FG loop from CD3γ into CD3δ, results in T cells whose 2C11 binding is virtually completely blocked by 17A2 (Fig. 2A, mutD; shaded curve). The completeness of this 17A2-mediated inhibition of 2C11-F binding is comparable to that observed in the CD3δ−/− T cells, indicating that 17A2 is able to bind to this mutant CD3εδ heterodimer (Fig. 2A, right panel). Further competitive binding experiments shown in Fig. 2B indicate that 17A2 inhibits 2C11 binding completely on αβ T cells from mutD mice, whereas in contrast, 17A2 inhibits 2C11 binding by only ~50% on αβ T cells from wt mice. Because murine γδ T cells lack CD3εδ, instead expressing two CD3εγ heterodimers per TCR complex (20), equivalent 2C11-F blocking activity by 17A2 on γδ T cells in both wt and mutD mice was expected and is observed (Fig. 2B).

Collectively, these findings show that the CD3γ G-strand segment is critical for 17A2 binding and that this segment can be introduced into CD3δ with no loss of TCR expression or function (Fig. 2). Such is not the case when residues in the CD3δ G-strand region are introduced into CD3γ (vide infra).

The CD3γ G-strand geometry is critical for TCR signaling

On the basis of the structural comparison and amino acid sequence alignment in Fig. 1, we generated a number of CD3γ mutants, in-
including CD3γ6M, a variant in which the six amino acids in the G-strand of CD3γ (ETSNPL) at positions 70–75 were replaced with those of CD3δ (KVSSSV) (Fig. 1A). This swap is predicted to foreclose the preceding segment and loop of the CD3γ6M mutant to adopt a more vertical trajectory than that of the CD3γwt counterpart. To test the functional significance of the CD3γ ectodomain modification, we exploited N15TCR tg mice specific for the vesicular stomatitis virus nuclear protein octapeptide (VSV8) bound to Kb (29, 36) and bred onto a CD3γ, and CD3δ chimera reactive with 17A2. These results support the notion that CD3γ6M adopts an extended G-strand more like mCD3δ, thereby preventing 17A2 mAb binding from the cleavage between CD3ε and CD3γ. Because the direct interaction residues mapped by proton-induced relaxation are exclusively in the CD3ε side (15), the trivial explanation that mutants abrogate 17A2 binding per se is excluded. Subsequently, we shortened the BC loop (LTDKT) in CD3γ by deleting the LTDKT sequence to create a second mutant, CD3γ6M-ΔBC. This foreshortened BC loop likely reinforces the upward trajectory of the FG loop in the CD3γ6M-ΔBC variant. Strikingly, the surface TCR expression rescue observed with CD3γ6M was not detected by any of the four Abs tested after CD3γ6M-ΔBC transduction. Collectively, these data suggest that this kinked CD3γ G-strand geometry is important for TCR surface expression, permitting CD3γε to occupy a position beneath the β FG loop of the β subunit and consistent with our previous TCR quaternary model (15) (Supplemental Fig. 2). In further support of this hypothesis, a third mutant in which the entire CD3γ ectodomain is replaced with that of CD3δ (CD3δmε6m) also fails to reconstitute TCR surface expression. Cellular protein expression for each of the mutant CD3γ transductants was comparable to that of wtCD3γ, as assessed by intracellular staining using the FLAG-tag appended to the Cyt tail of each construct and anti-FLAG Ab (Supplemental Fig. 3). However, we cannot exclude that in the case of CD3γ6M-ΔBC, incorrect protein folding may contribute to absent surface expression.

CD3γ AND CD3δ G-STRAND GEOMETRIES

Next, following retroviral transduction, N15 T cells were stimulated with VSV8-pulsed Kb-expressing APCs. As shown by the representative experiment in Fig. 3C, no significant IFN-γ production was obtained from GFP+ empty vector control transductants, whereas N15 T cells transduced with wtCD3γ cDNA showed specific responsiveness to VSV8 peptide in a concentration-dependent manner. N15 T cells expressing the CD3γ6M cDNA induced less IFN-γ production compared with that of CD3γwt, emphasizing the importance of the unique CD3γ G-strand geometry for Ag-triggered activation. As expected, VSV8 stimulation failed to induce IFN-γ production in N15 T cells transduced with CD3γ6M-ΔBC or CD3γmε6m.

Fig. 3D represents a composite histogram of IFN-γ production from multiple experiments using VSV8 stimulation of retrovirally transduced N15tgCD3γ−/− T cells. Both CD3γwt and CD3γ6M transductants (GFP+) produce IFN-γ upon VSV stimulation, but with cytokine production from CD3γwt greater than that from CD3γ6M transductants. Note the lack of detectable activation by pMHC in GFP− cells in either set of cultures. In contrast, with anti-CD3ε mAb triggering, IFN-γ production is observed in transduced (GFP+) and nontransduced (GFP−) populations, implying that 2C11 stimulation via CD3δε induces detectable IFN-γ production. Nonetheless, transduction of CD3γwt significantly augments that 2C11-stimulated response, compared with the vector control. Similar 2C11-stimulated IFN-γ production in CD3γwt and CD3γ6M transductants suggests that differential responsiveness to pMHC is not a consequence of TCR expression level. Together, the findings imply that TCRβ-CD3εy juxtaposition involving the kinked CD3γ G-strand affords an optimized geometry for effective Ag-triggered T cell activation.
expression on N15TCRtgCD3

These findings imply that the distinct topology of CD3 heterodimers assembly and signaling in the mammalian species. Furthermore, that TCRC among Gnathostamata (Fig. 4B).

The unique G-strand geometry of CD3γ is important for TCR complex surface expression and signaling. A. Schematic drawing of mCD3γ wt and CD3γ ε ectodomain mutant constructs. All constructs contain transmembrane and Cyt domains of mCD3γ, as well as a C-terminal FLAG-tag ( ). The mutation positions in CD3γ ectodomain are denoted in the accompanying ribbon diagrams (CD3ε, cyan; CD3γ, green; and CD3δ, yellow). B. Surface TCR expression on N15TCRtgCD3γwt T cells after CD3γ wt and CD3γ ε ectodomain mutant transduction. N15 T cells were stained with each indicated mAb and analyzed by flow cytometry after retroviral transduction. Data are representative of three independent experiments. C. pMHC/TCR-mediated signaling in N15TCRtgCD3γwt T cell transfectants. Transduced N15 T cells were stimulated with VSV8 peptide-loaded APCs for 4 h. The number of cytokine-producing cells was measured using flow cytometry after gating each GFP+CD8+ population in one representative experiment. D. Statistical analysis of VSV8- or 2C11-stimulated N15TCRtgCD3γwt transduced populations. Transduced T cells were stimulated by VSV8 peptide (1 μM) or plate-bound 2C11 Ab (5 μg/ml). GFP CD8+ and GFP+CD8+ populations were gated for IFN-γ+ analysis. The number of cytokine-producing cells was normalized by setting the IFN-γ amount from the CD3ε wt transduced N15TCRtgCD3γwt T cells as 1 and no stimulation as 0. Two-sided exact Wilcoxon rank-sum test was used to compare. Values are average ± SD (n = 3). *p ≤ 0.05; **p ≤ 0.01. TM, transmembrane.

Coevolution of the elongated Cβ FG loop and duplication of CD3γ plus CD3δ genes from a single precursor

Whereas the striking elongation of the Cβ FG loop among mammalian species is well conserved, sequence comparison with nonmammalian vertebrate species (chicken, fish, and frog) reveals that the lengthy Cβ FG loop is not observed in the latter (Fig. 4A). This rigid Cβ FG loop in mouse has been shown to facilitate both selection of thymocytes and activation of T cells (17, 18). The absence of distinct CD3γ and CD3δ subunits in the above-mentioned nonmammalian species and expression of a single precursor CD3γδ gene have been shown based on genomic and biochemical analyses, as well as theoretical predictions dating the required CD3 duplication event (37–39). Although recently it has been demonstrated that the jawless vertebrates (agnathans) have alternative adaptive immune systems with variable lymphocyte receptors, all jawed vertebrates (gnathostomates) possess fully developed adaptive immune systems with TCR and Ig genes (40). Both the elongated Cβ FG loop and the distinct CD3γ and CD3δ genes are unique in the mammalian species among Gnathostamata (Fig. 4B). These analyses support the notion that TCRβ and CD3γ have been evolutionarily coupled for TCR assembly and signaling in the mammalian species. Furthermore, these findings imply that the distinct topology of CD3 heterodimers coevolved with TRC domains to optimize the quaternary TCR structure for pMHC-triggered αβ TCR activation. TCRαβ heterodimer assembly studies with various CD3 complexes, using a phylogenetic approach with TCR complex subunits from mammalian and nonmammalian vertebrates, support this conclusion (41).

Restoration of T cell signaling and development by heterologous replacement of the mCD3γ ectodomain

Despite the above results and conclusions, it has been reported that hCD3δ can partially restore thymic development in the absence of mCD3γ in CD3γ-/- mice (42, 43), suggesting that the hCD3δ ectodomain may be accommodated in juxtaposition to the murine β subunit. We therefore assessed whether a chimeric CD3γ protein containing the hCD3δ ectodomain fused with transmembrane and Cyt tail segments of mCD3γ (CD3γhδecto) could restore TCR surface expression and T cell activation in N15tgCD3γ-/- T cells. In contrast to the inability of CD3γmδecto chimera to restore TCR expression and function, CD3γhδecto was competent to do both. As shown in Fig. 5A, CD3γhδecto transduction induced surface TCR expression comparable to that of CD3γwt, although, not surprisingly, with loss of 17A2 mAb reactivity. In vitro stimulation experiments likewise showed that CD3γhδecto possesses a signaling capacity equivalent to that of CD3γwt to induce IFN-γ production upon pMHC- (Fig. 5B) or 2C11- mediated stimulation
Thus, CD3γhδecto serves as a suitable structural surrogate for both VSV8- and 2C11-triggered activation.

To next assess whether this CD3γhδecto chimera can provide differentiation signals during development, an FTOC system was employed. Thymocyte progenitors from CD3γ2/2 fetal mice were transduced with CD3γwt or CD3γhδecto cDNA containing retroviruses of comparable viral titer. Subsequently, thymocytes generated within the reconstituted FTOC after 7 d of culture were

FIGURE 4. Coevolution of the elongated TCRβ FG loop and CD3γ plus CD3δ genes from a single precursor in Gnathostamata, a group of vertebrates with adaptive immunity involving a recombinatorial system (VDJ). A, Sequence comparison of the TCRβ FG loop regions among various species. The position of the F- and G-strands is defined based on the N15 TCR structure (16). The bracket region defines the elongated FG loop in mammalian species with well-conserved key residues (L219, W225, and P232) forming the hydrophobic core. The two cysteines contributing to the intrachain and interchain disulfide bond are indicated by d and s, respectively. The conserved lysine residue in the transmembrane region of Cβ is also highlighted in yellow.

B, Schematic representation of evolutionary relationships between TCRβ and CD3 gene products. Possession of adaptive immunity with recombinatorial-based immune receptors is known for agnathans and gnathostomates. Gnathostomates possess a developed adaptive immune system supporting various VDJ recombinations for Ig and TCR rearrangement, whereas Agnathans do not but contain variable lymphocyte receptors. Distinctions among features of mammals and those of birds, amphibians, reptiles, and bony fish are described and shown schematically.

FIGURE 5. Restoration of T cell signaling by replacing the ectodomain of mCD3γ with that of hCD3δ, but not mCD3δ. A, Surface TCR expression on N15TCRtgCD3γ−/− T cells resulting from chimeric CD3γ transduction. N15 T cells were stained with the indicated anti-TCR mAb for flow cytometric analysis after retroviral transduction with results of one of three representative experiments shown. B, pMHC/TCR-mediated signaling in N15 T cells. Transduced N15TCRtgCD3γ−/− T cells were stimulated with VSV8 peptide-loaded APCs for 4 h. The number of cytokine-producing cells was measured using flow cytometry after gating GFP+CD8+ populations. C, Anti-CD3ε mAb stimulation. Transduced N15 T cells were stimulated by plate-bound 2C11 Ab (5 µg/ml) for 3 h. GFP+ cells were separated by gating. The percentages of cells in the CD4SP, CD8SP, and DP quadrants are shown in the top panels. The percentages of cells in the DN3 (CD4−CD8−) and DN4 (CD4+CD8+) quadrants are shown in the middle panels after DN population (CD4−CD8−) gating. Histograms on the bottom panels show H57 staining for DN3 and DN4 subpopulations combined (CD4+CD8−CD44−).
prepared and analyzed for GFP expression and surface phenotype. As shown by CD4 and CD8 surface expression patterns in Fig. 5D (top), thymic development in the CD3-\(\gamma\delta\)ecto transduced thymocytes was equivalent or better than that of CD3\(\gamma\delta\) wt transduced thymocytes. In contrast to nontransduced cells (GFP+), the number of double positive thymocytes increased 2- to 5-fold. Fig. 5D (middle) shows that double negative (DN) cell development from DN1 (CD44+CD25+) plus DN2 (CD44+CD25+) stages to DN3 (CD44+CD25+) and DN4 (CD44+CD25+) stages was also accelerated by the transduction of CD3-\(\gamma\delta\)ecto. Thus, for example, in CD3-\(\gamma\delta\)ecto transductants, DN3 = 9.8% and DN4 = 42.2%, compared with 1.1 and 3.9%, respectively, for the GFP+ control. Furthermore, surface TCR\(\beta\) expression on DN3/4 stage cells was only slightly higher than on nontransduced cells (Fig. 5D, bottom), suggesting that pre-TCR expression is supported by CD3-\(\gamma\delta\)ecto as it is with wtCD3\(\gamma\) without nonphysiological overexpression of the pre-TCR. These results imply that the CD3-\(\gamma\delta\)ecto protein can replace that of the wtCD3\(\gamma\) component in the pre-TCR during T cell development.

Our findings with the chimeric CD3-\(\gamma\delta\)ecto protein are in agreement with results generated through transgenesis in CD3\(\gamma\)−/− mice using the hCD3\(\gamma\) (human only) construct (42, 43). Why, then, might these mouse-human interspecies CD3\(\gamma\delta\) heterodimers function to foster signaling (Fig. 6)? In this regard, it is noteworthy that the aromatic ring of Phe89 in human CD3\(\epsilon\) makes hydrophobic interactions with both the \(\gamma\)-methyl of Thr25 and the hydrophobic \(\beta\)-methylene position of the Ghn64 side chain of human CD3\(\epsilon\). This interaction creates a tightly packed interface between hCD3\(\epsilon\) and hCD3\(\epsilon\) molecules at the top of the G-G-strand interface (Supplemental Fig. 4). This preferential interaction would not be formed in the mCD3\(\epsilon\)-hCD3\(\epsilon\) dimer, as the Phe89 in hCD3\(\epsilon\) is a Thr in the corresponding position in mCD3\(\epsilon\). As a result, the top of the G-strand in CD3\(\gamma\delta\)ecto and hCD3\(\epsilon\) proteins, when dimerized with mCD3\(\epsilon\), can bend and slot into the area normally accommodating mCD3\(\gamma\)ecto in the TCR\(\beta\)-CD3\(\epsilon\)γ junction, without steric clash. Furthermore, hCD3\(\epsilon\) contains four alternating charged residues at the end of the FG loop (K57/D58/K59/E60). This charge cluster would destabilize a straight-up \(\beta\)-strand conformation with two positively charged lysines on one side and two negatively charged residues on the back side. Thus, the FG loop of hCD3\(\epsilon\) is most likely unstructured, consistent with its crystal structure (Supplemental Fig. 1) (35), and could be readily bent toward the back ABED face when heterodimerized with mCD3\(\epsilon\) in a fashion analogous to that of mCD3\(\gamma\). Differential glycosylation of mCD3\(\epsilon\) versus hCD3\(\epsilon\) with one fewer N-linked adduct in the human ortholog may also contribute to the functionality of this replacement.

Discussion

Analysis of CD3 sequence divergence indicates that CD3\(\gamma\), CD3\(\delta\), and CD3\(\epsilon\) genes arose from a common ancestor in a two-step process of gene duplication (38). Mammals have three CD3 genes (\(\gamma\), \(\delta\), and \(\epsilon\), whereas nonmammals (birds, fish, and amphibians) have only two: a CD3\(\gamma\) precursor and a CD3\(\epsilon\) gene. Protein sequence comparison indicates that each CD3\(\gamma\) and CD3\(\delta\) subunit evolved with highly homologous heterodimeric interfaces and membrane proximal segments for efficient and specific signaling transfer when paired with CD3\(\epsilon\). The compact orientation of the CD3\(\gamma\) FG loop and single horizontally attached glycan in the mouse is a feature of the CD3\(\gamma\) heterodimer that affords lateral support of the C region domains for \(\epsilon\) as well as \(\gamma\) TCRs. In addition, CD3\(\epsilon\) appears to have adapted to optimally interact with the C\(\beta\) FG loop. Our findings that the elongation of the structured C\(\beta\) FG loop coevolved with appearance of the CD3\(\gamma\) gene from a CD3\(\gamma\)δ precursor are strong support for this notion. By contrast, the more vertical CD3\(\epsilon\) FG loop trajectory and greater number of N-linked glycan adducts in CD3\(\delta\) heterodimers assume a more extended geometry that cannot fit into the homologous TCR\(\beta\)-CD3\(\epsilon\)γ interaction site (15). The CD3\(\delta\) disposition on the TCRs “side” of the complex occupies intervening space between the coreceptor (CD4 or CD8) and the \(\alpha\)β heterodimer (44, 45). This bulky CD3\(\delta\) component may also be entropically advantageous to help preconfigure the coreceptor as a TCR, pMHC, and coreceptor ternary complex forms.

The crystal structure of a \(\gamma\)δ TCR heterodimer reveals a C\(\gamma\)–C\(\delta\) domain symmetry, in contradistinction to the C\(\alpha\)–C\(\delta\) domain asymmetry observed in \(\alpha\)\(\beta\) TCRs (16, 46, 47). The \(\gamma\)δ TCR heterodimer also differs by lacking an elongated C\(\beta\) FG loop equivalent. \(\gamma\)δ TCR lineage commitment is associated with more robust signaling relative to that of the \(\alpha\)\(\beta\) TCR, with greater TCR copy number and/or ligand density likely affecting \(\gamma\)δ T lineage signaling strength (22, 48). In contrast, \(\alpha\)\(\beta\) TCR pMHC ligands are present at low levels, mandating additional TCR modifications to compensate for weak signals promoting \(\alpha\)\(\beta\) T cell fate and function. During immune surveillance, continued cell movement following ligation of the TCR \(\alpha\)\(\beta\) clonotype by specific pMHC fosters quaternary change; the C\(\beta\) FG loop interacts with CD3\(\epsilon\) on one side of the TCR and the C\(\delta\) domain with the bulky CD3\(\delta\) heterodimer on the other. It is the tangential rather than normal (i.e., perpendicular to the membrane) directional force that triggers TCR
activation post-pMHC ligation, as shown by optical tweezer experiments (15).

Our current results show how the αβ TCR quaternary structure is optimal for surface expression and signaling. Fig. 6A gives a side view of the surface-exposed TCR complex based upon existing structural information of individual components and molecular modeling (8, 15, 49). The substantial N-linked glycosylation of TCR subunits is indicated by the brown space-filling molecular representations. On the TCR ε “side,” the Cβ FG loop (17, 18) and the compact orientation of the Cδ3y FG loop (Fig. 1, Supplemental Fig. 2) are key features contributing to the asymmetry optimizing TCR signaling. Lateral movement of the TCR ε heterodimer can apply a torque on Cδ3y via the Cβ FG loop appendage. Fig. 6B schematically demonstrates that the extended Cδ3ε subunit ectodomain would sterically clash with the Cβ FG loop above, whereas that of Cδ3y or the chimeric heterodimer does not. On the TCR ε “side,” the bulky glycans and vertical Cδ3ε FG loop disposition may also likewise relay quaternary change to Cδ3y after tangential force-induced torque. Alternatively, the torque on Cδ3ε could be applied through the highly conserved connecting peptide at the base of the TCRεc domain (30). Further studies aimed at rigidifying or de-rigidifying segments of the TCR complex, without altering pMHC binding, should show an impact on TCR signaling, consistent with a mechanosensor mechanism of action.

The ability of the hCD3ε ectodomain to pair with mCD3ε and foster TCR complex expression signaling, as well as murine thymocyte development, might appear, at first glance, contradictory to our view that Cδ3y and Cδ3ε ectodomains evolved to occupy a different side of the TCR complex. However, that is not the case. Isologous subunit ectodomain substitution is not permitted, whereas the heterologous hCD3ε ectodomain can replace that of mCD3y. That functional substitution, as noted in Supplemental Fig. 4, is possible because mCD3ε has a Thr residue in lieu of Phe89 in hCD3ε, allowing the top of the G-strand of hCD3ε, when paired with mCD3ε, to avoid steric clash with the TCRβ subunit. We emphasize that the geometry of mCD3y and mCD3ε G-strand N-terminal residues (residues 70–75 and residues 58–63) are distinct from each other, as are the corresponding segments in the respective human orthologs.

As structural and functional analyses of these and other Ig-like domains of receptors become more sophisticated, additional subtleties and their biological implications will be revealed. The details as described in this study for CD3 heterodimers demonstrate the important functional consequences of structural evolution. Understanding these differences will help with elucidating the function of multisubunit receptors, such as the TCR.

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Disclosures
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References


