One of the CD3ε Subunits within a T Cell Receptor Complex Lies in Close Proximity to the Cβ FG Loop

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Summary

A recent crystal structure of the N15 α/β T cell receptor (TCR) in complex with an Fab derived from the H57 Cβ-specific monoclonal antibody (mAb) shows the mAb fragment interacting with the elongated FG loop of the Cβ domain. This loop creates one side wall of a cavity within the TCR Ti-α/β constant region module (CαCβ) while the CD and EF loops of the Cα domain form another wall. The cavity size is sufficient to accommodate a single nonglycosylated Ig domain such as the CD3ε ectodomain. By using specific mAbs to mouse TCR-β (H57) and CD3ε (2C11) subunits, we herein provide evidence that only one of the two CD3ε chains within the TCR complex is located in close proximity to the TCR Cβ FG loop, in support of the above notion. Moreover, analysis of T cells isolated from transgenic mice expressing both human and mouse CD3ε genes shows that the heterologous human CD3ε component can replace the mouse CD3ε at this site. The location of one CD3ε subunit within the rigid constant domain module has implications for the mechanism of signal transduction throughout T cell development.

Each TCR consists of a clonotypic TCR heterodimer (Ti-α/β or Ti-γ/δ subunits) in complex with the invariant CD3 chains (γ, δ, ε, and ζ). The disulfide-linked heterodimer represents the peptide-MHC ligand binding unit, thereby determining the ligand specificity of an individual T cell. In contrast, the CD3 polypeptides which are in noncovalent association with a given Ti heterodimer, mediate TCR base signal transduction (for review see references 1–5). Although CD3-γ and -δ are present in only one copy each (6–8), it appears that two copies of CD3ε and ζ exist per TCR complex (9–11). The signaling function of the CD3 components involves a conserved motif, termed an immunoreceptor tyrosine-based activation motif (ITAM) present in one to three copies in the cytoplasmic domain of each CD3 subunit (12, 13). The various CD3 subunits exhibit different interactions with intracellular signaling factors and induce distinct patterns of cellular protein tyrosine phosphorylation upon activation (14–19). How peptide-MHC ligand binding to a Ti-α/β or Ti-γ/δ heterodimer subsequently initiates signaling via the CD3 molecules is currently unknown.

Aside from their role in signal transduction, the CD3 subunits are also required for cell surface expression of the TCR heterodimers on mature T lymphocytes (20, 21), as well as for pre-T cell receptor function on immature CD4–CD8– double negative (DN) thymocytes (22, 23). Thus, without CD3ε or -ζ subunit expression there is a marked decrease or absence of TCR molecules on the cell surface as shown by in vitro analysis (20, 21, 24). In addition, in genetically engineered mouse strains in which these CD3 components are deleted by homologous recombination, there is a developmental blockade of thymocytes at the DN stage (25–29). The CD3δ subunit, in contrast to CD3ε and -ζ chains, is required for TCR expression only at a later stage of thymic development. The absence of CD3δ in a knockout mouse specifically blocks the thymic selection processes mediated by double positive (DP) (CD4+CD8+) to single positive (CD4+CD8− or CD4−CD8+) thymocytes (30).

Although the overall stoichiometry of the TCR complex is commonly given as TCR-α/β–CD3γδεζεζ, there is no direct structural evidence to support this subunit composition. Recently, a three-dimensional structure of the N15 vesicular stomatitis virus-specific/H-2Kb-restricted α/β-TCR (31) in complex with an Fab fragment from the
H57 anti-mouse Cβ-specific mAb (32) provided a clue with which to infer new details about the association between the TCR-α/β heterodimer and CD3e (33). We identified a cavity within the TCR-α/β C module formed by the Cβ FG loop, partially exposed Cβ domain strands, and conserved glycans from both Cα and Cβ domains that can accommodate a single Ig-like domain. Based on size and charge considerations, we suggested that the cavity probably represents the CD3e binding site. To determine whether there is a physical proximity between the Cβ FG loop and the CD3e chain, we performed a set of competition assays between the H57 and the CD3e-specific 2C11 mAbs (34). The results of these experiments provide evidence that one of the two CD3e subunits in a TCR complex is physically adjacent to the TCR-β constant region FG loop.

Materials and Methods

Transgenic Mice. Transgenic mice expressing the human CD3e gene (transgenic [tg] 600; reference 35) were provided by Dr. Cox Terhorst (Beth Israel Medical Center, Boston, MA) and are further referred to as hCD3e tg. This transgenic mouse strain contains 10–12 copies of the human CD3e transgene in a hemizygous mouse. Since T cell development is blocked in the homozygous mouse of this strain, we used hCD3e tg heterozygous mice for these studies. A littermate that does not contain this transgene was used as a control (wild-type, WT).

Molecular Modeling. The N15 TCR-H57 Fab complex (33). The three-dimensional structure of a complex crystal structure 3D coordinates (PDB code 1NFD; 33). The plot of the protein structure was created using the programs MOLSCRIPT (36) and RASTER3D (37).

Flow Cytometric Analysis. The following mAbs were used: R-PE-labeled anti-mouse CD3e (500A2; PharMingen, San Diego, CA), PE-labeled anti-mouse CD4 (H129.19; GIBCO BRL, Gaithersburg, MD), and Red613-labeled anti-mouse CD8 (53-6.7; GIBCO BRL). The mAbs hamster anti-mouse TCR-β (H57-57; reference 32), hamster anti-mouse CD3e (2C11-145; reference 34), and mouse anti-mouse Vβ8.1,2,3 (F23.1; reference 35) were purified and labeled with FITC using the Fluorescein Isothiocyanate labeling kit (Molecular Probes, Eugene, OR). Generation and purification of 2C11 and H57 Fab fragments were performed by using the ImmunoPure Fab Preparation Kit (Pierce, Rockford, IL). The purity of the Fab fragments was confirmed by SDS-PAGE, which demonstrated no residual intact IgG. Splenocytes from nontransgenic mice (WT) or mice heterozygous for human CD3e (hCD3e tg) were triple stained as follows: cells were incubated with PE-labeled anti-CD4, Red613-labeled anti-CD8, and FITC-H57 or FITC-2C11 for 30 min at 4°C in PBS containing 2% FCS. For mAb competition assays, splenocytes were first incubated with unlabeled H57 or 2C11 mAbs at 10-fold molar excess of the FITC-labeled mAb for 30 min at 4°C, washed with PBS + 2% FCS, and triple stained as explained above. Flow cytometric analysis was performed on a FACScan (Becton Dickinson, San Jose, CA). The mean fluorescence intensity (mFI) of the FITC-H57 or FITC-labeled 2C11 labeled T cells (CD4+ or CD8+ splenocytes) was measured and the FITC mFI of non-T cell splenocytes (CD4- or CD8-; background) was deducted from this value in each sample.

Results

A TCR Constant Domain Module Cavity as a Putative CD3e Binding Site. The three-dimensional structure of a complex between the N15 α/β-TCR and the anti-TCR-β mAb H57 Fab fragment resolved crystallographically to 2.8 Å has revealed several interesting features about TCR structure relative to antibody structure. As shown in Fig. 1, there is an obvious difference between the ligand binding surface of the V domain modules of the two receptors (Vαβ versus VhVl), with a relatively flat antigen binding surface in the case of the TCR, versus a concave surface in the case of the Fab. The flatness of the N15 antigen binding surface and the concavity of the H57 Fab surface are complementary to the surface of their respective ligands, Kβ and Cβ. Perhaps the most striking difference between these immunoreceptors is the symmetry of the Fab molecule compared with asymmetry of the TCR molecule. This TCR asymmetry results from the unusual arrangement of the Cα and Cβ domains relative to one another. Moreover, as shown in Fig. 1, the H57 Fab binds to the 12-residue Cβ FG loop conserved among TCR Cβ domains from multiple species (39). This loop architecture is uniquely rigidified by a hydrophobic minicore and an internal hydrogen bonding network. In addition, we have noted that this loop creates one side wall of a cavity, while the CD and EF loops of the Cα domain form the other side. The floor of the cave, which measures ~25 Å in depth, ~20 Å in height, and ~25 Å in width, is presumably formed by the plasma membrane on the T cell surface. The murine CD3e subunit, which is predicted to have an Ig fold (40), is nonglycosylated unlike CD3γ and CD3δ, and could readily fit into a cavity of this size.

Nonreciprocal Cross-blocking of 2C11 and H57 mAbs. To investigate our hypothesis that a CD3ε subunit might occupy this cavity within the C module and hence lie proximal to the Cβ FG loop, a set of competition assays between the H57 and 2C11 mAbs were performed using direct immunofluorescence analysis by FACS®. 2C11 was previously shown to specifically bind to the mouse CD3ε subunit (34). As shown in Fig. 2A, unlabeled competitor 2C11 or H57 was added before the addition of FITC-2C11, FITC-H57, or FITC-labeled anti-CD4, and log fluorescence was determined by quantitative FACS® analysis. As expected, unlabeled 2C11 blocks the subsequent binding of FITC-2C11 and, likewise, unlabeled H57 blocks the binding of FITC-H57. In contrast, neither unlabeled 2C11 nor H57 alters the binding of the unrelated FITC-H129.19. More importantly, note that under these conditions the anti-CD3ε mAb completely blocks the binding of FITC-H57 mAb, whereas H57 only partially reduces the mFI of FITC-2C11 binding to CD3ε on mouse splenic T cells. A second anti-CD3ε mAb, 500A2, also completely blocked FITC-H57...
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binding and, as with FITC-2C11, the binding of PE-500A2 was reduced by 50% when T cells were preincubated with unlabeled H57 (data not shown). Moreover, similar results were obtained by using 2C11 Fab or H57 Fab fragments for inhibition analysis. That the findings are not secondary to “general” steric blockade of the TCR by any mAb or Fab fragment binding to the complex is evident from the inability of Vβ-specific antibodies to block FITC-H57 binding on TCR transgenic T lymphocytes (data not shown).

Only one of the two CD3ε subunits is proximal to the Cβ FG Loop. To quantify the mFI reduction in FITC-2C11 resulting from unlabeled H57 preincubation in individual CD4 and CD8 T cell subsets, we performed the analogous competition experiments by three-color analysis, in addition using PE-labeled anti-mouse CD4 and Red613-labeled anti-mouse CD8. We collected data comparable to that shown in Fig. 2 A but on individual splenic CD4+ and CD8+ T lymphocytes. As shown in Fig. 2 B (left) for CD4+ T cells, although unlabeled 2C11 completely blocked the binding of FITC-2C11 to the mouse T cells, the unlabeled H57 mAb reduced the binding of the FITC-2C11 by ~50% (mFI, from 402 to 183). On the other hand, unlabeled 2C11 almost completely blocked the binding of FITC-H57 (mFI, from 398 to 45; Fig. 2 B, right). Identical results were obtained when CD8+ T cells subset were examined. Three conclusions may be drawn from these data. First, one CD3ε subunit is close to the H57 mAb binding site on the Cβ FG loop. Second, given that there are two CD3ε components per TCR (10, 11) and the H57 mAb can only block 50% of the FITC-2C11 binding, the second CD3ε subunit must exist at a distance from the Cβ FG loop. Third, this postulated TCR subunit arrangement is found in both CD4+ and CD8+ T cell subpopulations.

The Human CD3ε Subunit as well as the Mouse CD3ε Subunit Associates with the CαCβ Module. To test whether replacement of the murine CD3ε chain with the human CD3ε subunit might alter the binding affinity of the H57 mAb to the Cβ FG epitope, we used a well-characterized transgenic mouse strain (tg 600) engineered to express the human CD3ε component (reference 35; here referred to as hCD3εtg). Splenocytes from littermates that do not express the human CD3ε (WT), as well as a heterozygous hCD3εtg mouse, were stained with directly labeled FITC-2C11 mAb at concentrations ranging from 5 x 10-6 to 10-10 M.

As shown in Fig. 3 A, the hCD3εtg mouse expressed only

Figure 1. Model of the N15 α/β-TCR heterodimer complexed with the H57 anti-TCR Cβ-specific mAb Fab fragment. Based on x-ray coordinates and molecular modeling as described in Materials and Methods, a structure of the complex is shown. The TCR α chain is in red, the H57 Fab heavy chain is in gold, and H57 Fab light chain is in purple. The Cβ FG loop, which is the epitope recognized by the mAb H57, is indicated. This loop creates one side wall of a cavity (whose center is marked by the Cβ label) within the TCR Ti αβ constant region while the CD (*) and EF (**) loops of the Cα domain form the other side. The cavity size is sufficient to accommodate a single nonglycosylated Ig domain such as the CD3ε chain.
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However, 2C11 binding affinity to the mouse CD3ε subunit is not altered by the presence of the human CD3ε component (Kd ~10⁻⁸ M). The capacity of the unlabeled 2C11 mAb to block binding of FITC-H57 to heterozygous hCD3εtg T cells was also examined. As shown in Fig. 3B, the same amount of unlabeled 2C11 that blocks FITC-H57 binding to the WT mouse T cells only partially blocks the FITC-H57 binding to hCD3εtg T cells. This suggests that the human CD3ε subunit can replace the mouse CD3ε subunit in agreement with the finding of others (10, 41), and that it can occupy the cavity within the mouse TCR CβCACα module. Fig. 3B also demonstrates that the incubation of the mouse splenocytes with the 2C11 mAb does not block the binding of H57 to the TCR-β chain by downmodulation of the TCR complex from the T cell surface (42, 43). Thus, unlabeled 2C11 preincubation does not change the binding of the anti-Vβ8 mAb, FITC-F23.1, to T cells.

The Binding of the H57 mAb to the CβFG Loop Is Unaffected by Human Versus Mouse Origin of the CD3ε Component. In contrast to the results comparing mouse CD3ε in WT and hCD3εtg mice where the level of 2C11 expression is ~50% on the latter (Fig. 3A), H57 reactivity is equivalent in both (Fig. 4). This implies that the affinity of H57 mAb to the CβFG loop is not altered by the presence of the human CD3ε component. Had human CD3ε altered the affinity of H57, then the curves of mFI at different molar concentrations of FITC-H57 would have shown
a difference in the two mouse strains. Unlabeled 2C11 competitor mAb shifts the binding curve by ~2 logs at concentrations of FITC-H57 below $10^{-6}$ M. At the highest concentration of FITC-H57 ($10^{-6}$ M), the labeled antibody binds similarly in the presence or absence of unlabeled competitor 2C11 mAb. These findings are consistent with the notion that the epitopes defined by H57 and 2C11 mAbs are distinct but nevertheless partially overlapping.

**Discussion**

The crystal structure of the complex between the N15 TCR and an Fab fragment of the anti-Cβ mAb H57 led us to the hypothesis that one CD3ε may physically associate with the H57 mAb epitope on Cε (33). In this paper we demonstrate that one of the two CD3ε subunits of the TCR lies adjacent to the Cβ FG loop.

The overall shape of the TCR C domain module is remarkably asymmetric (Fig. 1). The Cβ domain bends more acutely towards the Vβ domain compared with the angle formed between the Cα and Vα domains. Cβ also has an unusually long and well-structured FG loop (the H57 mAb epitope) projecting down from the Vβ-Cβ interface in all TCRs studied to date (33, 44, 45). Although half of the Cβ domain’s ABED sheet is surface exposed, it does not make contact with the Cα domain. As described in detail by Wang et al. (33), this asymmetry creates a cave-like structure or cavity below the β chain sufficient in size to accommodate a single Ig domain. The partially exposed ABED β sheet of the Cβ domain forms an extensive ceiling. The CD and EF loops of the Cα domain, along with the glycans attached to CαN 185, CαN 121, and CβN 186 form one side wall, and the FG loop of Cβ and the glycan emanating from CβN 236 form the other side wall of the cavity. The glycans project outward and do not obstruct the cavity. The floor of the cave is presumably formed by the plasma membrane on the T cell surface (33). It is noteworthy that the accessible surface at the α/β-TCR cavity contains multiple basic residues.

The murine CD3ε subunit consists of 87 residues in the extracellular segment, and has Ig-like characteristics suggesting that this segment can readily fold into a small Ig domain (40). CD3ε is the only CD3 subunit with a nonglycosylated Ig-like ectodomain in the TCR complex of man and mouse (40). Moreover, the CD3ε subunit has twice as many acidic residues as basic ones and is therefore negatively charged (pl = 4.5, whereas the predicted pls of the CD3γ and CD3ζ extracellular domains are more basic, being 8.76 and 5.82, respectively). The charge complementation between the acidic residues of the CD3ε subunit and the basic cavity also argues in favor of the CD3ε subunit occupying this site. The initial hypothesis regarding the proximity of CD3ε and the Cβ FG loop was confirmed here by a set of competition assays in which the 2C11 and H57 mAbs, which bind to epitopes on CD3ε and TCR-β, respectively, were able to alter each other’s binding capacity. We further showed that although there are two CD3ε subunits per TCR-β subunit, only one of the CD3ε components is in close proximity to the Cβ FG loop. Given that the γδ TCR includes CD3 components (46) and is predicted to contain an equivalent insertion in the corresponding constant domain loop (47) analogous to the Cβ FG loop, we can reasonably predict that a cavity with a comparable arrangement for CD3ε exists in γδ T cells.

If the TCR C module cavity associates with one CD3ε, where is the second CD3ε located? In vitro translation studies have shown that when CD3ε is translated alone it tends to form disulfide-linked oligomers. However, using more physiological TCR-α/β-CD3 expression conditions, namely simultaneous cotranslation of all the TCR subunits, the cotranslation of CD3γ or CD3ζ was sufficient to keep CD3ε in a monomeric state (48). Both CD3γ and CD3ζ compete for binding to CD3ε (49). In addition, it was shown that the CD3γ/ε pair associates with TCR-ε, whereas the CD3γ/ζ pair associates with TCR-β. This association takes place upon formation of an intrachain disulfide bridge between TCR-ε and -β (10). Additional studies have shown that CD3ε and TCR-β pair via their ectodomains, whereas the association of the other CD3 components with TCR-ε and -β is largely mediated by interactions within their transmembrane regions. The proximity of TCR-β and CD3γ has been suggested by cross-linking experiments in humans (50) and mice (7). Based on these findings, it is likely that the CD3ε subunit that is physically associated with the Cβ FG loop is paired with CD3γ and that the second CD3ε, which pairs with the extracellular domain of the CD3ζ subunit, associates via its transmembrane domain with the TCR-ε (51). The ability of the CD3ε/γ dimer–specific mAb 7D6 (52) to partially inhibit FITC-H57 binding to T cells is also consistent with this view (data not shown).

An interesting feature of the potential interaction of CD3ε within the TCR C domain module relates to certain
critical Cα residues that are preserved in pre-Tα (pTα), a 30-kD glycoprotein whose expression is restricted to early CD4+CD8− DN lineage cells (53–55). The pre-TCR, a disulfide-linked heterodimer of a functionally rearranged TCR-β chain and the pTα chain, noncovalently associated with the CD3 components is expressed in DN immature thymocytes (56). During development, the DN to DP thymocyte transition is induced by an as yet unknown ligand that binds to the pre-TCR, resulting in expression of mature type αβ TCR heterodimers on DP thymocytes (57–59). Although there is only 12% identity between pTα and Cα in humans and mice (60), the Cα residues involving important polar interactions with the Cβ domain are all conserved in the pTα sequence. Given that conserved structural elements account for close to half of the pTα–Cα amino acid sequence identities, it is very obvious that the heterodimer interface of pTα–Cβ will be extremely similar to that of Cα–Cβ. With this in mind and since genetic disruption of the CD3ε gene results in T cell development blockage at the DN stage, it is likely that the CD3ε subunit is accommodated in the pre-TCR module in a manner similar to its mode in the mature αβ-TCR.

One can imagine that certain mouse CD3ε residues contribute to the contacts made between H57 and the TCR. In this regard, the total buried molecular surface area at the N15 TCR-H57 Fab interaction is 1460 Å² (720 Å² for N15 and 740 Å² for H57) (33), consistent with the range of values observed for other intact protein antigen–Fab interactions (61–63). However, one unusual feature of the N15–H57 interaction is the predominance of contacts made by overall loop orientation relative to the Vα domain. With this in mind and since genetic disruption of the CD3ε gene results in T cell development blockage at the DN stage, it is likely that the CD3ε subunit can fit into the mouse TCR C domain module cavity (Fig. 3B). Given that the extracellular domain of the mouse and human CD3ε share 53% identity in their amino acid sequence, it is possible that any putative CD3ε residues that interact with the H57 heavy chain are conserved among mouse and human CD3ε.

Although the precise functional role of the FG loop in Cβ is far from clear, it is likely to have an important impact on signaling and/or TCR assembly and structure. The unique FG loop is conserved among sequences of mouse, rat, human, and rabbit TCRs (39). Although absent in TCRs sequences from some other species, it is replaced by a potential glycosylated addition site whose glycan may serve a similar structural function (33). Undoubtedly this loop influences the mobility and disposition of Vβ relative to Cβ domains. Although the minihydrophobic patch of this loop as resolved from the crystal structure fixes the overall loop orientation relative to the Vβ and Cβ domains, local movements are permitted (33). How, if at all, peptide-MHC interactions might transfer information to the CD3 signaling subunits by affecting the FG Cβ loop remains to be tested experimentally.

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