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Stoichiometry of the T cell Antigen Receptor (TCR) Complex: Each TCR/CD3 Complex Contains One TCR α, One TCR β, and Two CD3ε Chains

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Summary

The stoichiometry of the subunits that comprise the T cell antigen receptor (TCR) complex is not completely known. In particular, it is uncertain whether TCR α and TCR β proteins are present in the TCR complex as one or multiple heterodimeric pairs. In this study we have used mice transgenic for two different TCR α and two different TCR β proteins to determine the number of TCR α and TCR β chains in a single TCR complex. Individual thymocytes and splenic T cells from double TCR transgenic mice simultaneously expressed all four transgenic TCR proteins on their surfaces. Because the individual TCR α and individual TCR β proteins were biochemically distinguishable, we were able to examine associations among the transgenic TCR products. We found that each TCR α chain paired with each TCR β chain, but that each TCR complex contained only one TCR α and one TCR β protein. Furthermore, quantitative immunofluorescence revealed that T cells expressed twice as many CD3ε as TCR β proteins. These findings demonstrate that there are precisely one TCR α, one TCR β, and two CD3ε chains in each TCR/CD3 complex expressed on the surfaces of both thymocytes and mature T cells.

The TCR, a heterodimer of α and β proteins, is expressed on the surface of T lymphocytes as part of a multisubunit complex that includes the CD3 proteins (CD3γ, δ, and ε) and a dimer of the ζ protein family (1). Although all members of the surface TCR/CD3 complex have been identified, their stoichiometry has not yet been completely determined. Recent studies showing that at least two, and possibly only two, CD3ε proteins are present per TCR/CD3 complex have led to a reevaluation of TCR/CD3 structure and the proposal that CD3 components exist in a single complex as two dimers of γ/ε and δ/ε proteins (2–4). Several groups have also raised the possibility that there are two TCR α and two TCR β chains in a single TCR complex (5, 6), arguing that such an arrangement would facilitate signal transduction after TCR engagement by antigen and would result in an electrostatically neutral and, hence, more stable protein complex.

To determine whether there were more than one TCR α or TCR β protein in a single TCR/CD3 complex, we examined associations among components of surface TCR on thymocytes and splenic T cells coexpressing two different TCR α and two different TCR β chains. The results of these experiments provide a unique solution to the stoichiometry of the TCR/CD3 complex and demonstrate that there are only one TCR α chain, one TCR β chain, and two CD3 ε chains in a single TCR/CD3 complex.

Materials and Methods

Mice. Mice transgenic for the AND TCR α and β chains (7) were originally provided by S. Hedrick (University of California at San Diego, La Jolla, CA) and were bred in our facility. They had been backcrossed for more than 10 generations onto a C57BL/6-Ly5.2 background. Mice transgenic for the anti-H-Y TCR α and β chains (8) were originally provided by H. von Boehmer (Basel Institute, Basel, Switzerland) and were bred in our facility. Double transgenic mice were generated by crossing a mouse heterozygous for the AND transgenes with a mouse heterozygous for the anti-H-Y transgenes. Female single and double TCR transgenic animals were used in these studies.

Antibodies. Biotinylated mAbs anti-Vα11 (RR8-1 [9]) and anti-Vβ3 (KJ-25 [10]) are specific for the AND TCR α and TCR β chains, respectively, were purchased from PharMingen (San Diego, CA). T3.70, the mAb specific for the anti-H-Y TCR α chain Vα3, was provided by H. von Boehmer (8) and conjugated to FITC in our lab. mAb anti-Vβ8.1, 8.2, 8.3 (F23.1 [11]) binds the anti-H-Y TCR β chain and was purified and conjugated to FITC in our lab. mAb anti-CD3ε (145-2C11 [12]) was used as a hybridoma supernatant for precipitations and as a protein A-purified preparation for quantitative surface staining. Both mAb anti-CD3ε (500A2 [13]) and mAb anti-TCR β (H57-597 [14]) were also used as protein A-purified preparations for quantitative surface staining.

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Cells. Thymi were dissected from young adult (6–8 wk) mice. Single cell suspensions of thymocytes were prepared by gently teasing cells from the thymic capsule and filtering over nylon mesh. Splenic T cells were prepared by panning murine splenocytes on plates coated with rabbit anti-mouse Ig (Organon Teknika, Durham, NC) to rid the population of B cells and adherent cells. Splenic CD4+ (CD8-depleted) T cells were prepared from C57BL/6 mice by further treating the cells with anti-CD8 (3.155 [15]), anti-NK1.1 (PK136 [16]), anti-I-A\(^{d,q}\) (M5/114.15.2 [17]) and rabbit complement for 30 min at 37°C, and then with rabbit complement alone for another 30 min at 37°C. Viable cells were collected after centrifugation over Lympholyte-M (Cedarlane Laboratories, Ltd., Hornby, Canada).

Staining. Murine thymocytes and splenic T cells (5 x 10^6) were stained with saturating concentrations of biotinylated and FITC-conjugated reagents in 40 μl for 30 min at 4°C, washed in 200 μl staining medium (HBSS, 0.1% BSA, 0.1% NaN3) three times, then stained at 2°C with saturating concentrations of Texas Red streptavidin for 15 min at 4°C. Cells were washed again and analyzed on a FACSstar Plus® (Becton Dickinson & Co., Mountain View, CA). For quantitative immunofluorescence of splenic T cells, 10⁶ cells were washed and incubated at 4°C for 1 h with unlabeled anti-TCR β (H57-597), unlabeled anti-CD3ε (145-2C11), and 1.0 μg/ml for 500A2) at concentrations determined empirically to be saturating. Because these antibodies share the same isotype (hamster IgG), their relative binding intensities could be compared directly. After extensive washing, bound antibodies were visualized with FITC-conjugated goat anti-hamster antibody, which was also used at saturating concentrations.

Surface Iodination, Immunoprecipitation, and Endoglycosidase-F (Endo F) Digestion. Cells were pelleted, washed with cold PBS and treated with Bolton Hunter reagent (18) before they were surface iodinated as described previously (19). Surface iodinated cells were lysed in 1% digitonin as described previously (19) and immunoprecipitated with beads preadsorbed to fetal calf serum, then with beads preadsorbed to 2–4 μg purified antibody (anti-Vα11, anti-Vβ3) or 1 ml hybridoma supernatant (F23.1, T3.70, and 2C11). Immunoprecipitates were washed in buffer containing 0.2% digitonin (18) and digested overnight with Endo F/Peptide-N-Glycosidase F (Endo F/PNGase F) (Oxford GlycoSystems, Rodsdale, NY) at 37°C, as per the manufacturer's instructions.

Two-dimensional NEPHGE/SDS-PAGE Electrophoresis. Immunoprecipitated samples were equilibrated in 150 μl sample buffer (9.5 M urea, 2% Triton X-100, 1.6% Ampholyte 5-7 [Bio-Rad Laboratories, Richmond, CA], 0.4% Ampholyte 3-10 [Bio-Rad Laboratories], 5% 2-mercaptoethanol) and separated in tube gels across a pH gradient (nonequilibrium pH gradient electrophoresis [NEPHGE]) for 2500 volt-hours as described previously (18, 20). Tube gels were extruded, equilibrated at 37°C in SDS-PAGE sample buffer containing 2-mercaptoethanol and run in the second dimension on a 13% reducing PAGE gel. Gels were run for 865 Vh, fixed, dried, and visualized by phosphorimagery.

Results

Experimental Design. The stoichiometry of the TCR/CD3 complex has not yet been solved and a number of TCR/CD3 configurations are consistent with current data (Fig. 1). Various solutions differ by the number of TCR α and β chains present per complex and the ratio between the moles of TCR β (or TCR α) and CD3 ε represented in each complex. Models proposing more than one TCR α and TCR β in a single complex predict that, on a cell expressing two different TCR α chains, antibodies specific for one TCR α would coprecipitate the other TCR α. Similarly, on cells expressing two different TCR β chains, antibodies specific for one TCR β would coprecipitate the other TCR β chain. In the present study, we have tested this prediction by generating cells that coexpressed two different TCR α and two different β chains on their surfaces and have examined associations among complex components. The usefulness of such an approach, however, required that the two different TCR α and two different TCR β proteins be biochemically distinguishable.

The AND TCR α and TCR β Chains Are Biochemically Distinguishable from the H-Y TCR α and TCR β Chains. We compared the biochemical signatures of the TCR α and β proteins expressed by mice transgenic for the AND TCR α/β (7) with the biochemical signatures of the TCR α and β chains expressed by mice transgenic for the male antigen H-Y (8) (hitherto referred to as the H-Y TCR α/β receptor). Neither the AND and H-Y TCR α chains (\(^{\text{AND}}V\alpha11\) and \(^{\text{H-Y}}V\alpha3\)) nor the AND and H-Y TCR β chains (\(^{\text{AND}}V\beta3\) and \(^{\text{H-Y}}V\beta8\)) could be distinguished by molecular weight or charge (data not shown). However, when stripped of their carbohydrate side chains with Endo F/PNGase F, which hydrolyze N-linked sugar residues, and then separated by charge, the two α chains and the two β chains exhibited unique migration patterns when resolved by two-dimensional NEPHGE under reducing conditions (Fig. 2). The \(^{\text{AND}}V\alpha11\) chain migrated as two spots, designated α111 and α112 (Fig. 2) and the \(^{\text{H-Y}}V\alpha3\) chain migrated as a significantly more acidic single spot, designated α3. The
Figure 2. Deglycosylated AND and H-Y TCR α chains and the AND and H-Y TCR β chains are biochemically distinguishable via two dimensional NEPHGE/SDS-PAGE analysis. Thymocytes from AND transgenic and H-Y transgenic mice were surface-labeled with 125I, lysed in digitonin, and precipitated with mAbs indicated. Precipitates were digested with Endo F, then resolved by NEPHGE followed by SDS-PAGE under reducing conditions, as indicated. Where indicated, precipitates from AND and H-Y lysates were mixed just before electrophoresis. Separated proteins were visualized by phosphorimaging.

**AND/Vα3**

AND Transgenic Thymocytes and Splenocytes Coexpress AND and H-Y TCR α and TCR β Proteins on Their Surfaces. Because the AND and H-Y receptor proteins could be distinguished biochemically, we generated T cells that coexpress both receptors by crossing mice transgenic for the AND TCR α/β receptor with mice transgenic for the H-Y TCR α/β. Immature and mature T lymphocytes in double TCR transgenic progeny expressed all four transgenic TCR chains (AND/Vα11, H-Y/Vα3 [T3.70], AND/Vβ3, and H-Y/Vβ8) on their surfaces simultaneously (Fig. 3). Surface staining revealed that the large majority of thymocytes in double transgenic mice bound antibodies to both AND/Vα3 and H-Y/Vβ8 as well as both AND/Vα11 and H-Y/Vα3 (Fig. 3, bottom left).

**AND/H-Y Double TCR Transgenic Thymocytes and Splenocytes**

Figure 3. Thymocytes and splenocytes from double TCR transgenic mice coexpress both TCR α and TCR β chains on their surfaces. Single cell suspensions of thymocytes and splenic T cells from single and double TCR transgenic littermates were stained simultaneously with biotinylated antibodies specific for Vα11 and fluorescein-conjugated antibodies specific for H-Y/Vα3 (T3.70) or with biotinylated antibodies specific for AND/Vα3 and fluorescein conjugated antibodies specific for H-Y/Vβ8. Cells were washed, incubated with Texas Red streptavidin, and analyzed on a FACStar Plus®. Quadrants define the boundaries of negative control profiles.
Immunoprecipitations supported the indication that AND and H-Y TCR proteins were expressed on the surface of the same cells. Antibodies to ANDVα11 coprecipitated both ANDVα3 and H-Vα8, as shown by the appearance of the β31, β32, and the spot unique to H-Y, β82 (Fig. 4 A, left, see arrow to the right). Reciprocally, ANDVα3 coprecipitated both ANDVα11 and H-Vα3 from double TCR transgenic thymocytes, as indicated by the presence of the α111, α112, and the spot unique to H-Y, α3 (Fig. 4 A, right, see arrow on the left). Hence, TCR α and TCR β chains of different transgenic origins shared the same receptor complex, indicating (a) that they were expressed on the surface of the same cell and (b) that the two transgenic TCR α chains could freely associate with each of the two transgenic TCR β chains.

Thymocytes and Splenic T Cells Express Only One TCR α and One TCR β per TCR/CD3 Complex. As expected, anti-CD3ε (2C11) coprecipitated both ANDVα3 and ANDVα11 from single AND transgenic thymocytes and precipitated both H-Vα8 and H-Vα3 from single H-Y transgenic thymocytes. Anti-CD3ε also coprecipitated all transgenic TCR chains from the surface of AND/H-Y double transgenic thymocytes as indicated by the presence of β81 and β82 spots and by the presence of α111, α112, and α3 spots (Fig. 4 A, right).

Although the AND TCR β and the H-Y TCR β chains associated with both transgenic TCR α chains and with CD3ε, they never associated with each other. Antibodies to ANDVα11 failed to coprecipitate H-Vα3 from the surface of double transgenic thymocytes, for the spot unique to H-Vα3, i.e., α3, was absent after NEPHGE analysis (Fig. 4 A, left, left arrow) Anti-H-Vα3 (T3.70) also failed to precipitate spots unique to ANDVα11 (data not shown). Similarly, the two transgenic TCR β chains did not associate with each other, for anti-ANDVα3 failed to precipitate the spot unique to H-Vα8, i.e., β82 (Fig. 4 A, middle, right arrow).

Thus, these data indicated that ANDVα11 and H-Vα3 were both expressed on the surface of double transgenic thymocytes but were not present in the same complex. Likewise, both ANDVα3 and H-Vα8 were present on the surface of double TCR transgenic thymocytes, but were not present in the same complex. Therefore, each TCR complex expressed by double TCR transgenic thymocytes contained only one TCR α and one TCR β chain.

To address the possibility that stoichiometry of TCR complexes differed between immature and mature T cells, we examined the associations of transgenic TCR α chains expressed by splenic T cells from single (AND) and double (AND/H-Y) transgenic mice (Fig. 4 B). Anti-Vα11 precipitated ANDVα11 from the surfaces of both AND single transgenic and AND/H-Y double transgenic splenic T cells, as shown by the appearance of spots α111 and α112. However, anti-Vα11 did not coprecipitate H-Vα3, for the α3 spot was absent by NEPHGE analysis. As was observed in thymocytes, these findings demonstrate that ANDVα11 was not present in the same TCR complex that contained H-Vα3. We conclude that there is only one TCR α per complex expressed by mature T cells.

The TCR/CD3 Complex Contains One TCR α, One TCR β, and Two CD3ε Proteins. Our findings rule out models of TCR/CD3 stoichiometry that propose more than one TCR α and one TCR β chain per complex (Fig. 1, B and C) and also predict that CD3ε chains outnumber TCR β chains on the surface of T cells (Fig. 1 A). To quantify the relative expression levels of CD3ε and TCR β proteins on normal T cells, we compared the densities of TCR β and CD3ε on normal splenic T cells by immunofluorescence. When incubated with saturating concentrations of anti-TCR β (H57-597) or with saturating concentrations of two different anti-CD3ε antibodies (either 145-2C11 or 500A2), all of which share the
Figure 5. There is a 1:2 ratio of binding sites for anti-TCR β and anti-CD3ε on the surface of T cells. Splenic CD4+ T cells were prepared and stained for quantitative immunofluorescence as described under Materials and Methods. Total fluorescent units (FU) × 10^-4 are displayed for each curve in parentheses. FU = cell frequency × median intensity and median intensity is derived by converting median logarithmic channel numbers to linear units via a calibration curve empirically derived for each logarithmic amplifier used. Negative control antibody fluorescence (lightly shaded) is subtracted.

Discussion

In the present study we assessed the stoichiometry of the TCR α and TCR β chains in the TCR/CD3 complex expressed on the surface of immature and mature T cells. When associations between different TCR α and different TCR β chains of T lymphocytes from mice transgenic for two different TCR α/β heterodimers were examined, it was clear that each of the transgenic TCR α proteins freely associated with each transgenic TCR β protein, and vice versa. However, no more than one TCR α and TCR β was ever detected in a single TCR complex. Antibodies specific for one transgenic TCR α chain never coprecipitated the other transgenic TCR α chain and, reciprocally, antibodies against one transgenic TCR β chain never coprecipitated the other transgenic TCR β chain. The absence of any association between either the transgenic TCR α protein or the transgenic TCR β chains directly rules out the existence of TCR complexes containing multiple independent TCR α/β pairs. The present data even rule out models that postulate TCR complexes containing multiple TCR α/β pairs where different individual TCR α or different individual TCR β chains are constrained from sharing the same complex. For example, even if the two transgenic TCR α proteins (H-YVα8 and ANDVα3) were constrained from sharing a single complex, each anti-Vα antibody would have coprecipitated the other transgenic TCR β protein because each transgenic TCR α chain paired freely with both transgenic TCR β chains. Similarly, if the two transgenic TCR β proteins (H-YVβ8 and ANDVβ3) were incompatible, each anti-Vβ antibody would have coprecipitated the other transgenic TCR α protein because each transgenic TCR β chain paired freely with both transgenic TCR α chains. The only possibility that our data do not preclude is that TCR complexes on double transgenic mice exclusively contain multiple identical TCR α/β pairs. Such a possibility would require V-region incompatibilities not only between the two transgenic TCR α proteins in different disulfide linked α/β dimers, but also between the two transgenic TCR β proteins. It is difficult to imagine a physical basis for such a severe constraint. Thus, we conclude that each TCR complex contains only one TCR α and one TCR β chain (Fig. 1A).

Quantitative surface staining further demonstrates that CD3ε and TCR β are expressed on the surface of normal T cells at a 2:1 ratio, as proposed in Fig. 1A. Because the anti-CD3ε antibody 2C11 recognizes CD3ε in association with either CD3γ or CD3δ, there are two ε containing dimers per TCR complex (2γε, 2δε, or one of each), a stoichiometry previously advocated by the data and conclusions of de la Hera et al. (4). However, the precise stoichiometry of CD3δ, CD3γ, and ζε proteins still remains unknown. We cannot reconcile our data with studies suggesting that CD3δ and TCR β are present on the surface of T cells in a 1:1 ratio (21, 22), since our immunofluorescent assessment clearly indicates that TCR β and CD3ε proteins are present in a 1:2 ratio. Therefore, the present study demonstrates that there are precisely one TCR α, one TCR β and two CD3ε proteins in a single TCR/CD3 complex.

What made it possible for us to assess associations between the two different TCR α and the two different TCR β chains in double transgenic mice were the distinctive patterns each transgenic protein generated after they were stripped of their N-linked oligosaccharides before NEPHGE analysis. Without Endo F/PNGase digestion, the TCR proteins were indistinguishable, although H-YVα3 was slightly more acidic than ANDVα3 (data not shown). Every transgenic TCR protein except H-YVα3 resolved into several spots after digestion. These biochemical signatures were remarkably reproducible and multiple additions of fresh Endo F/PNGase did not change the pattern of spots generated. The multiple spots formed by each TCR α and TCR β chain during NEPHGE analysis presumably represent the variety of TCR protein products generated when mixed Endo F/PNGase activities act upon a pool of heterogeneously glycosylated TCR α and β chains. It could also be a reflection of heterogeneity in accessibility of individual chains to Endo F/PNGase digestion.

The possibility that there were more than one TCR α/β heterodimer per complex was originally raised to account for a number of observations, the most compelling of which was the recognition that an α/β/ε ratio of 1:1:2 would result in a transmembrane charge imbalance (5). Because TCR α and TCR β chains have three positively charged transmembrane residues between them and the CD3 proteins and ζ chains each have one negatively charged transmembrane residue, a TCR/CD3 complex with one TCR α/β, two CD3es, one
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Our findings offer an explanation also proposed by de la Hera et al. (4) for data demonstrating a difference in the developmental and functional effects of anti-CD3ε and anti-TCR β (24–27). Because there are two CD3ε chains per complex, anti-CD3ε is capable of aggregating multiple receptor complexes and generating intracellular signals through multivalent cross-linking of TCR/CD3 components. However, because there is only one TCR β chain per TCR complex anti-TCR β can induce no more than bivalent TCR cross-linking. The signals transduced by bivalent rather than multivalent cross-linking may be quantitatively and qualitatively distinct.

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Stoichiometry of the TCR/CD3 Complex

1 dimer, one CD3ε, and one CD3γ would have a net charge of -3. It is possible that as yet unidentified proteins and/or lipids associate with the complex to maintain charge neutrality. A charge imbalance may, in fact, give the TCR/CD3 complex the flexibility to interact with different surface proteins at different times during activation or differentiation. Alternatively, arithmetic calculations of net charge may not take into account complexities introduced by tertiary structure which may mute the imbalance. It is also possible that charge neutrality is not critical for stability of surface proteins. Indeed, the neu oncogene product is stably expressed on the cell surface despite a net negative charge of -1 within its transmembrane domain (23).
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