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Jennifer Punt  
*Haverford College*

Joseph L. Roberts

Kelly P. Kearse

Alfred Singer

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## Stoichiometry of the T cell Antigen Receptor (TCR) Complex: Each TCR/CD3 Complex Contains One TCR $\alpha$ , One TCR $\beta$ , and Two CD3 $\epsilon$ Chains

By Jennifer A. Punt, Joseph L. Roberts, Kelly P. Kearse, and Alfred Singer

From the Experimental Immunology Branch, National Cancer Institute, Bethesda, Maryland 20892

### 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  $\alpha$  and TCR  $\beta$  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  $\alpha$  and two different TCR  $\beta$  proteins to determine the number of TCR  $\alpha$  and TCR  $\beta$  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  $\alpha$  and individual TCR  $\beta$  proteins were biochemically distinguishable, we were able to examine associations among the transgenic TCR products. We found that each TCR  $\alpha$  chain paired with each TCR  $\beta$  chain, but that each TCR complex contained only one TCR  $\alpha$  and one TCR  $\beta$  protein. Furthermore, quantitative immunofluorescence revealed that T cells expressed twice as many CD3 $\epsilon$  as TCR  $\beta$  proteins. These findings demonstrate that there are precisely one TCR  $\alpha$ , one TCR  $\beta$ , and two CD3 $\epsilon$  chains in each TCR/CD3 complex expressed on the surfaces of both thymocytes and mature T cells.

The TCR, a heterodimer of  $\alpha$  and  $\beta$  proteins, is expressed on the surface of T lymphocytes as part of a multisubunit complex that includes the CD3 proteins (CD3 $\gamma$ ,  $\delta$ , and  $\epsilon$ ) and a dimer of the  $\zeta$  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, CD3 $\epsilon$  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  $\gamma/\epsilon$  and  $\delta/\epsilon$  proteins (2–4). Several groups have also raised the possibility that there are two TCR  $\alpha$  and two TCR  $\beta$  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  $\alpha$  or TCR  $\beta$  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  $\alpha$  and two different TCR  $\beta$  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  $\alpha$  chain, one TCR  $\beta$  chain, and two CD3  $\epsilon$  chains in a single TCR/CD3 complex.

### Materials and Methods

**Mice.** Mice transgenic for the AND TCR  $\alpha$  and  $\beta$  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  $\alpha$  and  $\beta$  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. Progeny were screened for the expression of transgenic TCR products by examining PBL for surface expression of the AND TCR  $\alpha$  (<sup>AND</sup>V $\alpha$ 11) and the anti-H-Y TCR  $\beta$  (<sup>H-Y</sup>V $\beta$ 8) chains. Female single and double TCR transgenic animals were used in these studies.

**Antibodies.** Biotinylated mAbs anti-V $\alpha$ 11 (RR8-1 [9]) and anti-V $\beta$ 3 (KJ-25 [10]) are specific for the AND TCR  $\alpha$  and TCR  $\beta$  chains, respectively, were purchased from PharMingen (San Diego, CA). T3.70, the mAb specific for the anti-H-Y TCR  $\alpha$  chain V $\alpha$ 3, was provided by H. von Boehmer (8) and conjugated to FITC in our lab. mAb anti-V $\beta$ 8.1, 8.2, 8.3 (F23.1 [11]) binds the anti-H-Y TCR  $\beta$  chain and was purified and conjugated to FITC in our lab. mAb anti-CD3 $\epsilon$  (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-CD3 $\epsilon$  (500A2 [13]) and mAb anti-TCR  $\beta$  (H57-597 [14]) were also used as protein A-purified preparations for quantitative surface staining.

**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<sup>+</sup> (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<sup>b,d,q</sup> (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 ( $5 \times 10^5$ ) were stained with saturating concentrations of biotinylated and FITC-conjugated reagents in 40  $\mu$ l for 30 min at 4°C, washed in 200  $\mu$ l staining medium (HBSS, 0.1% BSA, 0.1% NaN<sub>3</sub>) three times, then stained in 40  $\mu$ l with saturating concentrations of Texas Red streptavidin for 15 min at 4°C. Cells were washed again and analyzed on a FACStar Plus<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA). For quantitative immunofluorescence of splenic T cells,  $10^6$  cells were washed and incubated at 4°C for 1 h with unlabeled anti-TCR  $\beta$  (H57-597), unlabeled anti-CD3 $\epsilon$  (145-2C11 or 500A2) at concentrations determined empirically to be saturating (0.5  $\mu$ g/ml for H57-597, 0.25  $\mu$ g/ml for 145-2C11, and 1.0  $\mu$ g/ml for 500A2). 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.

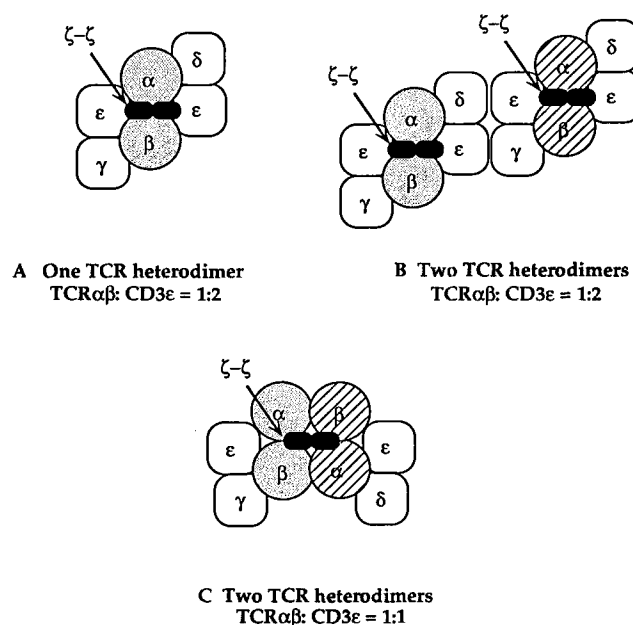
**Surface Iodination, Immunoprecipitation, and Endoglycosidase-F (Endo) F Digestion.** Cells were pelleted, washed with cold PBS and treated with Bolton Hunter reagent (18) before they were surface iodinated as described previously (19). Surface iodinated cells were lysed in 1% digitonin as described previously (19) and immunoprecipitated with beads preadsorbed to fetal calf serum, then with beads preadsorbed to 2–4  $\mu$ g purified antibody (anti-V $\alpha$ 11, anti-V $\beta$ 3) 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, Rosedale, NY) at 37°C, as per the manufacturer's instructions.

**Two-dimensional NEPHGE/SDS-PAGE Electrophoresis.** Immunoprecipitated samples were equilibrated in 150  $\mu$ l 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 phosphorimager.

## 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). Var-

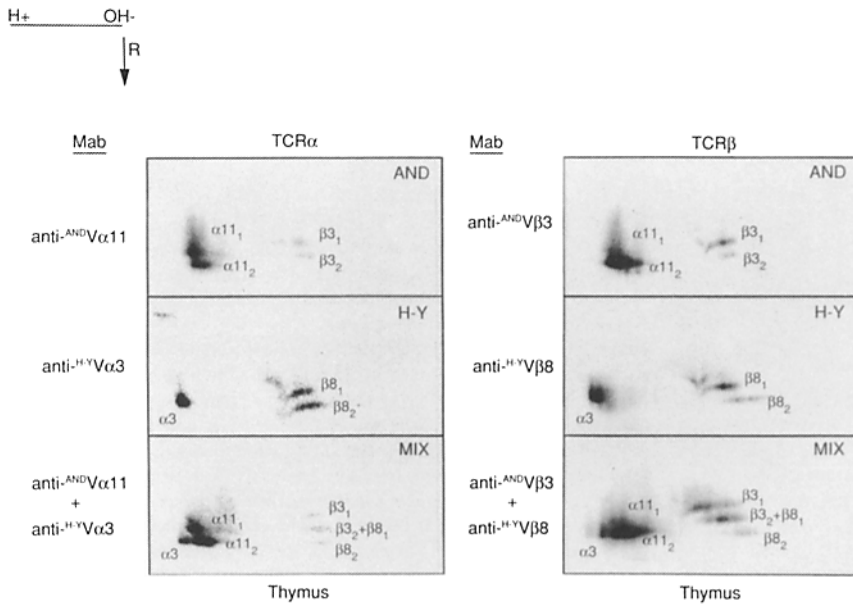
<sup>1</sup> Abbreviations used in this paper: Endo F, Endoglycosidase F; NEPHGE, nonequilibrium pH gradient electrophoresis; PNGase F, Peptide-N-Glycosidase F.



**Figure 1.** Three models of TCR/CD3 stoichiometry.

ious solutions differ by the number of TCR  $\alpha$  and  $\beta$  chains present per complex and the ratio between the moles of TCR  $\beta$  (or TCR  $\alpha$ ) and CD3  $\epsilon$  represented in each complex. Models proposing more than one TCR  $\alpha$  and TCR  $\beta$  in a single complex predict that, on a cell expressing two different TCR  $\alpha$  chains, antibodies specific for one TCR  $\alpha$  would coprecipitate the other TCR  $\alpha$ . Similarly, on cells expressing two different TCR  $\beta$  chains, antibodies specific for one TCR  $\beta$  would coprecipitate the other TCR  $\beta$  chain. In the present study, we have tested this prediction by generating cells that coexpressed two different TCR  $\alpha$  and two different  $\beta$  chains on their surfaces and have examined associations among complex components. The usefulness of such an approach, however, required that the two different TCR  $\alpha$  and two different TCR  $\beta$  proteins be biochemically distinguishable.

**The AND TCR  $\alpha$  and TCR  $\beta$  Chains Are Biochemically Distinguishable from the H-Y TCR  $\alpha$  and TCR  $\beta$  Chains.** We compared the biochemical signatures of the TCR  $\alpha$  and  $\beta$  proteins expressed by mice transgenic for the AND TCR  $\alpha/\beta$  (7) with the biochemical signatures of the TCR  $\alpha$  and  $\beta$  chains expressed by mice transgenic for the receptor specific for the male antigen H-Y (8) (hitherto referred to as the H-Y TCR  $\alpha/\beta$  receptor). Neither the AND and H-Y TCR  $\alpha$  chains (ANDV $\alpha$ 11 and H-YV $\alpha$ 3) nor the AND and H-Y TCR  $\beta$  chains (ANDV $\beta$ 3 and H-YV $\beta$ 8) 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  $\alpha$  chains and the two  $\beta$  chains exhibited unique migration patterns when resolved by two-dimensional NEPHGE under reducing conditions (Fig. 2). The ANDV $\alpha$ 11 chain migrated as two spots, designated  $\alpha$ 1<sub>1</sub> and  $\alpha$ 1<sub>2</sub> (Fig. 2) and the H-YV $\alpha$ 3 chain migrated as a significantly more acidic single spot, designated  $\alpha$ 3. The



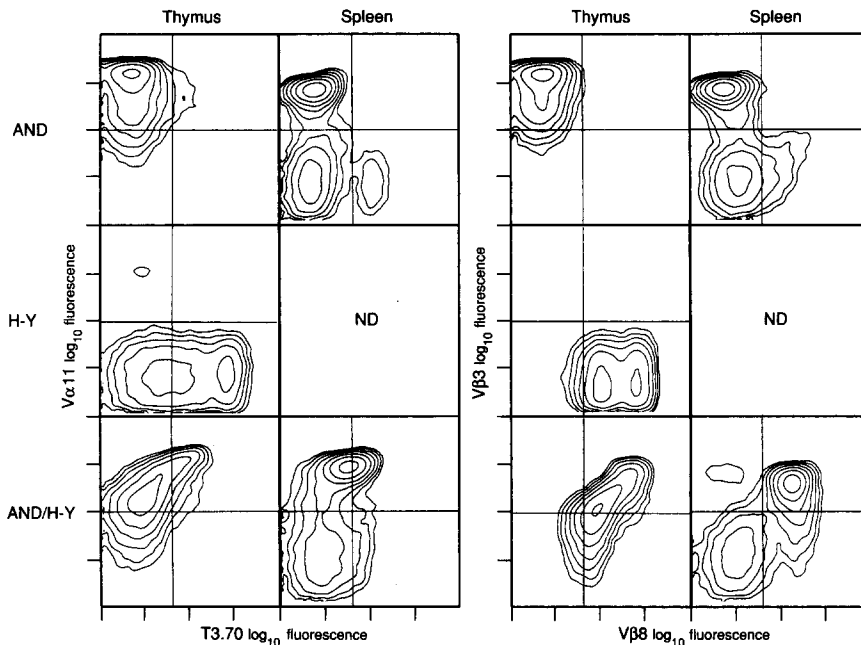
**Figure 2.** Deglycosylated AND and H-Y TCR  $\alpha$  chains and the AND and H-Y TCR  $\beta$  chains are biochemically distinguishable via two dimensional NEPHGE/SDS-PAGE analysis. Thymocytes from AND transgenic and H-Y transgenic mice were surface-labeled with  $^{125}\text{I}$ , lysed in digitonin, 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 phosphorimager.

$\text{ANDV}\beta 3$  chain migrated as two major spots, designated  $\beta 3_1$  and  $\beta 3_2$ , as did the  $\text{H-YV}\beta 8$  chain, designated  $\beta 8_1$  and  $\beta 8_2$ . The  $\beta 8_2$  spot generated by  $\text{H-YV}\beta 8$  was significantly lower in molecular weight than any spot generated by  $\text{ANDV}\beta 3$ .

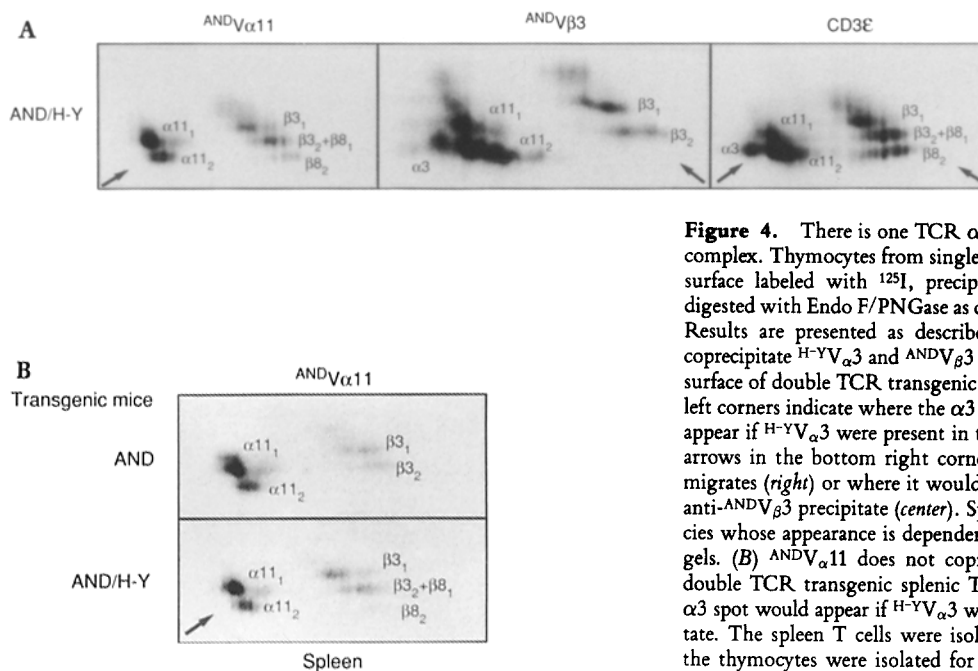
Most importantly, the two TCR  $\alpha$  and the two TCR  $\beta$  chains were distinguishable from each other when anti-TCR  $\alpha$  or anti-TCR  $\beta$  precipitations from AND and H-Y thymocytes were mixed together before NEPHGE analysis. In particular, the appearance of the  $\alpha 3$  and  $\beta 8_2$  spots, unique to  $\text{H-YV}\alpha 3$  and  $\text{H-YV}\beta 8$ , respectively, provided unambiguous evidence for the presence of the H-Y transgene products in immunoprecipitated preparations (Fig. 2).

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

*cytes Coexpress AND and H-Y TCR  $\alpha$  and TCR  $\beta$  Proteins on Their Surfaces.* Because the AND and H-Y receptor proteins could be distinguished biochemically, we generated T cells that coexpressed both receptors by crossing mice transgenic for the AND TCR  $\alpha/\beta$  receptor with mice transgenic for the H-Y TCR  $\alpha/\beta$ . Immature and mature T lymphocytes in double TCR transgenic progeny expressed all four transgenic TCR chains ( $\text{ANDV}\alpha 11$ ,  $\text{H-YV}\alpha 3$  [T3.70],  $\text{ANDV}\beta 3$ , and  $\text{H-YV}\beta 8$ ) on their surfaces simultaneously (Fig. 3). Surface staining revealed that the large majority of thymocytes in double transgenic mice bound antibodies to both  $\text{ANDV}\beta 3$  and  $\text{H-YV}\beta 8$  as well as both  $\text{ANDV}\alpha 11$  and  $\text{H-YV}\alpha 3$  (Fig. 3, bottom left).



**Figure 3.** Thymocytes and splenocytes from double TCR transgenic mice coexpress both TCR  $\alpha$  and TCR  $\beta$  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  $\text{V}\alpha 11$  and fluorescein-conjugated antibodies specific for  $\text{H-YV}\alpha 3$  (T3.70) or with biotinylated antibodies specific for  $\text{ANDV}\beta 3$  and fluorescein conjugated antibodies specific for  $\text{H-YV}\beta 8$ . Cells were washed, incubated with Texas Red streptavidin, and analyzed on a FACStar Plus<sup>®</sup>. Quadrants define the boundaries of negative control profiles.



**Figure 4.** There is one TCR  $\alpha$  and one TCR  $\beta$  chain per TCR/CD3 complex. Thymocytes from single and double TCR transgenic mice were surface labeled with  $^{125}\text{I}$ , precipitated with the indicated mAbs, and digested with Endo F/PNGase as described under Materials and Methods. Results are presented as described in Fig. 2. (A)  $\text{ANDV}\alpha_{11}$  does not coprecipitate  $\text{H-YV}\alpha_3$  and  $\text{ANDV}\beta_3$  does not coprecipitate  $\text{H-YV}\beta_8$  from the surface of double TCR transgenic thymocytes. The arrows in the bottom left corners indicate where the  $\alpha_3$  spot migrates (right) or where it would appear if  $\text{H-YV}\alpha_3$  were present in the anti- $\text{ANDV}\alpha_{11}$  precipitate (left). The arrows in the bottom right corners indicate either where the  $\beta_{8_2}$  spot migrates (right) or where it would migrate if  $\text{H-YV}\beta_8$  were present in the anti- $\text{ANDV}\beta_3$  precipitate (center). Spots that are not labeled are minor species whose appearance is dependent on the extent of the exposure of the gels. (B)  $\text{ANDV}\alpha_{11}$  does not coprecipitate  $\text{H-YV}\alpha_3$  from the surface of double TCR transgenic splenic T cells. The arrow indicates where the  $\alpha_3$  spot would appear if  $\text{H-YV}\alpha_3$  were present in the anti- $\text{ANDV}\alpha_{11}$  precipitate. The spleen T cells were isolated from the same mice from which the thymocytes were isolated for the studies shown in A.

Immunoprecipitations supported the indication that AND and H-Y TCR proteins were expressed on the surface of the same cells. Antibodies to  $\text{ANDV}\alpha_{11}$  coprecipitated both  $\text{ANDV}\beta_3$  and  $\text{H-YV}\beta_8$ , as shown by the appearance of the  $\beta_{3_1}$ ,  $\beta_{3_2}$ , and the spot unique to H-Y,  $\beta_{8_2}$  (Fig. 4 A, left, see arrow to the right). Reciprocally,  $\text{ANDV}\beta_3$  coprecipitated both  $\text{ANDV}\alpha_{11}$  and  $\text{H-YV}\alpha_3$  from double TCR transgenic thymocytes, as indicated by the presence of the  $\alpha_{11_1}$ ,  $\alpha_{11_2}$ , and the spot unique to H-Y,  $\alpha_3$  (Fig. 4 A, middle, see arrow on the left). Hence, TCR  $\alpha$  and TCR  $\beta$  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  $\alpha$  chains could freely associate with each of the two transgenic TCR  $\beta$  chains.

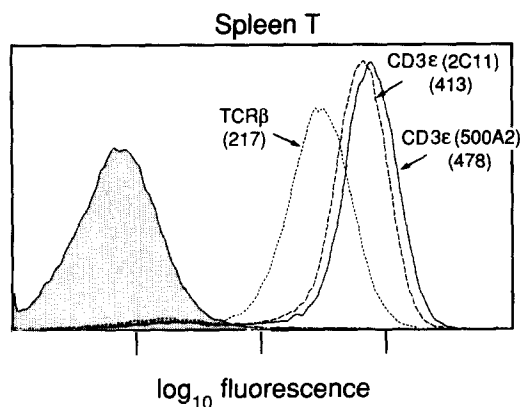
**Thymocytes and Splenic T Cells Express Only One TCR  $\alpha$  and one TCR  $\beta$  per TCR/CD3 Complex.** As expected, anti-CD3 $\epsilon$  (2C11) coprecipitated both  $\text{ANDV}\beta_3$  and  $\text{ANDV}\alpha_{11}$  from single AND transgenic thymocytes and precipitated both  $\text{H-YV}\beta_8$  and  $\text{H-YV}\alpha_3$  from single H-Y transgenic thymocytes. Anti-CD3 $\epsilon$  also coprecipitated all transgenic TCR chains from the surface of AND/H-Y double transgenic thymocytes as indicated by the presence of  $\beta_{8_1}$  and  $\beta_{8_2}$  spots and by the presence of  $\alpha_{11_1}$ ,  $\alpha_{11_2}$ , and  $\alpha_3$  spots (Fig. 4 A, right).

Although the AND TCR  $\beta$  and the H-Y TCR  $\beta$  chains associated with both transgenic TCR  $\alpha$  chains and with CD3 $\epsilon$ , they never associated with each other. Antibodies to  $\text{ANDV}\alpha_{11}$  failed to coprecipitate  $\text{H-YV}\alpha_3$  from the surface of double transgenic thymocytes, for the spot unique to  $\text{H-YV}\alpha_3$ , i.e.,  $\alpha_3$ , was absent after NEPHGE analysis (Fig. 4 A, left, left arrow). Anti- $\text{H-YV}\alpha_3$  (T3.70) also failed to precipitate spots unique to  $\text{ANDV}\alpha_{11}$  (data not shown). Similarly, the two transgenic TCR  $\beta$  chains did not associate with each other, for anti- $\text{ANDV}\beta_3$  failed to precipitate the spot unique to  $\text{H-YV}\beta_8$ , i.e.,  $\beta_{8_2}$  (Fig. 4 A, middle, right arrow).

Thus, these data indicated that  $\text{ANDV}\alpha_{11}$  and  $\text{H-YV}\alpha_3$  were both expressed on the surface of double transgenic thymocytes but were not present in the same complex. Likewise, both  $\text{ANDV}\beta_3$  and  $\text{H-YV}\beta_8$  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  $\alpha$  and one TCR  $\beta$  chain.

To address the possibility that stoichiometry of TCR complexes differed between immature and mature T cells, we examined the associations of transgenic TCR  $\alpha$  chains expressed by splenic T cells from single (AND) and double (AND/H-Y) transgenic mice (Fig. 4 B). Anti- $\text{V}\alpha_{11}$  precipitated  $\text{ANDV}\alpha_{11}$  from the surfaces of both AND single transgenic and AND/H-Y double transgenic splenic T lysates, as shown by the appearance of spots  $\alpha_{11_1}$  and  $\alpha_{11_2}$ . However, anti- $\text{V}\alpha_{11}$  did not coprecipitate  $\text{H-YV}\alpha_3$ , for the  $\alpha_3$  spot was absent by NEPHGE analysis. As was observed in thymocytes, these findings demonstrate that  $\text{ANDV}\alpha_{11}$  was not present in the same TCR complex that contained  $\text{H-YV}\alpha_3$ . We conclude that there is only one TCR  $\alpha$  per complex expressed by mature T cells.

**The TCR/CD3 Complex Contains One TCR  $\alpha$ , One TCR  $\beta$ , and Two CD3 $\epsilon$  Proteins.** Our findings rule out models of TCR/CD3 stoichiometry that propose more than one TCR  $\alpha$  and one TCR  $\beta$  chain per complex (Fig. 1, B and C) and also predict that CD3 $\epsilon$  chains outnumber TCR  $\beta$  chains on the surface of T cells (Fig. 1 A). To quantify the relative expression levels of CD3 $\epsilon$  and TCR  $\beta$  proteins on normal T cells, we compared the densities of TCR  $\beta$  and CD3 $\epsilon$  on normal splenic T cells by immunofluorescence. When incubated with saturating concentrations of anti-TCR  $\beta$  (H57-597) or with saturating concentrations of two different anti-CD3 $\epsilon$  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  $\beta$  and anti-CD3 $\epsilon$  on the surface of T cells. Splenic CD4<sup>+</sup> T cells were prepared and stained for quantitative immunofluorescence as described under Materials and Methods. Total fluorescent units (FU)  $\times 10^{-4}$  are displayed for each curve in parentheses. FU = cell frequency  $\times$  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.

same isotype (hamster IgG) and subsequently incubated with the same fluorescently tagged secondary antibody (goat anti-hamster IgG), splenic T cells stained with anti-CD3 $\epsilon$  fluoresced with greater intensity than those stained with anti-TCR  $\beta$  (Fig. 5). When total fluorescence units were compared, precisely twice as much anti-CD3 $\epsilon$  bound the surface of splenic T cells as anti-TCR  $\beta$ , indicating that CD3 $\epsilon$  and TCR  $\beta$  were present at a 2:1 ratio on the T cell surface.

## Discussion

In the present study we assessed the stoichiometry of the TCR  $\alpha$  and TCR  $\beta$  chains in the TCR/CD3 complex expressed on the surface of immature and mature T cells. When associations between different TCR  $\alpha$  and different TCR  $\beta$  chains of T lymphocytes from mice transgenic for two different TCR  $\alpha/\beta$  heterodimers were examined, it was clear that each of the transgenic TCR  $\alpha$  proteins freely associated with each transgenic TCR  $\beta$  protein, and vice versa. However, no more than one TCR  $\alpha$  and TCR  $\beta$  was ever detected in a single TCR complex. Antibodies specific for one transgenic TCR  $\alpha$  chain never coprecipitated the other transgenic TCR  $\alpha$  chain and, reciprocally, antibodies against one transgenic TCR  $\beta$  chain never coprecipitated the other transgenic TCR  $\beta$  chain. The absence of any association between either the transgenic TCR  $\alpha$  protein or the transgenic TCR  $\beta$  chains directly rules out the existence of TCR complexes containing multiple independent TCR  $\alpha/\beta$  pairs. The present data even rule out models that postulate TCR complexes containing multiple TCR  $\alpha/\beta$  pairs where different individual TCR  $\alpha$  or different individual TCR  $\beta$  chains are constrained from sharing the same complex. For example, even if the two transgenic TCR  $\alpha$  proteins (<sup>H-Y</sup>V $\alpha$ 3 and <sup>AND</sup>V $\alpha$ 11) were constrained from sharing a single complex, each anti-V $\beta$  antibody would have coprecipitated the other transgenic TCR  $\beta$  protein because

each transgenic TCR  $\alpha$  chain paired freely with both transgenic TCR  $\beta$  chains. Similarly, if the two transgenic TCR  $\beta$  proteins (<sup>H-Y</sup>V $\beta$ 8 and <sup>AND</sup>V $\beta$ 3) were incompatible, each anti-V $\alpha$  antibody would have coprecipitated the other transgenic TCR  $\alpha$  protein because each transgenic TCR  $\beta$  chain paired freely with both transgenic TCR  $\alpha$  chains. The only possibility that our data do not preclude is that TCR complexes on double transgenic mice exclusively contain multiple identical TCR  $\alpha/\beta$  pairs. Such a possibility would require V-region incompatibilities not only between the two transgenic TCR  $\alpha$  proteins in different disulfide linked  $\alpha/\beta$  dimers, but also between the two transgenic TCR  $\beta$  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  $\alpha$  and one TCR  $\beta$  chain (Fig. 1 A).

Quantitative surface staining further demonstrates that CD3 $\epsilon$  and TCR  $\beta$  are expressed on the surface of normal T cells at a 2:1 ratio, as proposed in Fig. 1 A. Because the anti-CD3 $\epsilon$  antibody 2C11 recognizes CD3 $\epsilon$  in association with either CD3 $\gamma$  or CD3 $\delta$ , there are two  $\epsilon$  containing dimers per TCR complex (2 $\gamma\epsilon$ , 2 $\delta\epsilon$ , 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 CD3 $\delta$ , CD3 $\gamma$ , and  $\zeta$  proteins still remains unknown. We cannot reconcile our data with studies suggesting that CD3 $\epsilon$  and TCR  $\beta$  are present on the surface of T cells in a 1:1 ratio (21, 22), since our immunofluorescent assessment clearly indicates that TCR  $\beta$  and CD3 $\epsilon$  proteins are present in a 1:2 ratio. Therefore, the present study demonstrates that there are precisely one TCR  $\alpha$ , one TCR  $\beta$  and two CD3 $\epsilon$  proteins in a single TCR/CD3 complex.

What made it possible for us to assess associations between the two different TCR  $\alpha$  and the two different TCR  $\beta$  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 <sup>H-Y</sup>V $\alpha$ 3 was slightly more acidic than <sup>AND</sup>V $\alpha$ 11 (data not shown). Every transgenic TCR protein except <sup>H-Y</sup>V $\alpha$ 3 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  $\alpha$  and TCR  $\beta$  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  $\alpha$  and  $\beta$  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  $\alpha/\beta$  heterodimer per complex was originally raised to account for a number of observations, the most compelling of which was the recognition that an  $\alpha/\beta/\epsilon$  ratio of 1:1:2 would result in a transmembrane charge imbalance (5). Because TCR  $\alpha$  and TCR  $\beta$  chains have three positively charged transmembrane residues between them and the CD3 proteins and  $\zeta$  chains each have one negatively charged transmembrane residue, a TCR/CD3 complex with one TCR  $\alpha/\beta$ , two CD3 $\epsilon$ s, one

$\zeta$  dimer, one CD3 $\delta$ , and one CD3 $\gamma$  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).

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-CD3 $\epsilon$  and anti-TCR  $\beta$  (24–27). Because there are two CD3 $\epsilon$  chains per complex, anti-CD3 $\epsilon$  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  $\beta$  chain per TCR complex anti-TCR  $\beta$  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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Address correspondence to Dr. J. Punt, Experimental Immunology Branch, National Cancer Institute, Building 10, Room 4B-17, Bethesda, MD 20892.

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