Structure of the T Cell Antigen Receptor (TCR): Two CD3ε Subunits in a Functional TCR/CD3 Complex

By Antonio de la Hera, Urs Müller, Carina Olsson, Sylvie Isaaz, and Alan Tunnacliffe

From the Basel Institute for Immunology, CH-4005 Basel, Switzerland

Summary

Transgenic mice carrying and expressing the human CD3ε gene incorporate the corresponding protein product into T cell receptor (TCR)/CD3 complexes on thymocyte and T cell surfaces. The chimeric antigen receptors allow normal T cell development and selection of repertoires in vivo and are able to transduce activation signals in vitro. We have exploited the ability to distinguish mouse (m) and human (h)CD3ε chains to analyze the stoichiometry of CD3ε in transgenic mouse TCRs. Immunoprecipitation and fluorescence resonance energy transfer experiments demonstrate that such TCRs can contain both h- and mCD3ε chains, implying that more than one CD3ε subunit occurs per TCR. Antigen comodulation studies are consistent with a stochastic use of h- or mCD3ε during receptor assembly, and further suggest a structure for the TCR/CD3 complex with two CD3ε chains. The determination of CD3ε subunit stoichiometry, together with existing biochemical data, allows the generation of a minimal model for the structure of the TCR and illustrates the potential value of the transgenic approach to the analysis of complex receptors.

TCRs for antigen (TCR/CD3) comprise two types of component: (a) two variable subunits (TCR-α/β or -γ/δ) that specifically bind antigen; and (b) several invariable chains that are noncovalently associated with TCR chains (1, 2). The invariant chains are implicated in the assembly and regulation of surface expression of the TCR/CD3 complex (3–8), as well as in the transduction of antigen-driven activation signals (3, 8–11), and consist of the products of the CD3 gene family itself (CD3γ, CD3δ, CD3ϵ), and the ζ and η chains, which are structurally unrelated to CD3 (12–17).

In spite of the rapid progress in our knowledge about the individual subunits, we have limited information about the quaternary structure of the compound receptor. The general view has been that the subunit stoichiometry of TCR/CD3 complex is either $\alpha\beta\gamma\delta\epsilon\zeta_2$ or $\alpha\beta\gamma\delta\epsilon\zeta\eta$ (18–20). However, we are not aware of any previous study that accurately quantitates the number of individual chains, and therefore, data supporting the above representation of TCR/CD3 subunits are lacking.

It is difficult to establish the stoichiometry of compound receptors in which associations are via noncovalent bonds, as occurs for many of the subunits of the TCR/CD3 complex. We reasoned that one method of calculating the multiplicity of individual subunits might be to study receptors that bear polymorphic forms of a given subunit, which can thus be experimentally distinguished. Transgenic mice facilitate such an approach when the introduced gene is polymorphic with respect to its endogenous counterpart. Here,

we have exploited interspecies polymorphism and analyzed CD3ε subunit stoichiometry using T cells from human CD3ε transgenic mouse strains (21). We report that both mouse and human CD3ε chains can be present in the same TCR/CD3 complex, on T cells that have undergone normal TCR selection in vivo and bear surface receptors that transduce activation signals in vitro, indicating that the TCR contains two CD3ε subunits.

Material and Methods

Transgenic Mice. A preliminary description of transgenic mice strains carrying and expressing the human CD3ε gene has been published (21), and further details will be given elsewhere (Olsson, C., et al., manuscript in preparation). Briefly, five independent strains were constructed by injection of BDF2 fertilized eggs with NotI-linearized cosmid (cε1; 13) DNA. The initial backcross of founder mice was with the C57BL/6 inbred strain, and positive hemizygous offspring were subjected to brother/sister mating. Resultant homozygotes were maintained as established transgenic lines from founder mice with low transgene copy numbers (TG16, one copy; TG21, three copies; TG41, five copies). High-copy strains (TG22: $\sim$40 copies; TG31: $\sim$90 copies) produced homozygotes infrequently, and in most cases, these mice did not thrive. During the establishment of the TG strains, we observed that 7 of 10 of hemizygous mice carrying high-copy (i.e., $\sim$40 or 90) and 1 of 16 having low-copy (i.e., one, three, or five) numbers of the transgene had a small spleen and a thymus that was barely detectable. Accordingly, thymocyte numbers were severely reduced to $\sim$2 $\times$ 10^6/thymus (range, 1–8 $\times$ 10^6/thymus), while controls contained

80–100 × 10^6/thymus. The spleen contained about half the normal T cell numbers. Both thymocytes and spleen T cells in such immunodeficient mice showed significantly reduced levels of both mouse (m)[1]- m and human (h) CD3ε on the cell surface (our unpublished observations). Although the immunodeficiency was not strictly related to copy number or transgene integration site, its prevalence in the high-copy mice may be at least partially responsible for our difficulties in establishing viable homozygotes from these lines. The immunodeficient phenotype was not observed in the homozygous transgenic mice, described in this report.

Antibodies. We used mAb specific for either human antigens CD3ε (UCHT1; 22) and CD2 (Leu-5b; Becton Dickinson & Co., Mountain View, CA [B-D]); mouse antigens CD3ε (145-2C11; 23), L3T4 (mCD4; B-D), Lyt-2 (mCD8; B-D), Thy-1 (B-D), I-E^d (14.4.4; American Type Culture Collection, Rockville, MD), panTCR-β (H57-597; 24), TCR-V$_{γ3}$ (536; 25), TCR-V$_{β11}$ (RR3-15; 26), TCR-V$_{β6}$ (RR4-7; 27), and TCR-V$_{β8}$ (F23.1; 28); and antisera specific for either human, mouse, or hamster Ig (Southern Biotechnology Associates, Birmingham, AL). Where indicated, antibodies were conjugated with either FITC, tetramethylrhodamine isothiocyanate (TRITC), or biotin, as described elsewhere (29).

Cell Lines, Proliferation, and Cytotoxicity Assays. JM and B6.2.16 BW2.1 are human and mouse T cell lines, respectively; U937 is an Ig FcR-bearing human histiocytoma. T cell lines from either transgenic and control littermate mouse or human origin were prepared as previously described (21). The proliferation and ^{51}Cr release cytotoxicity assays were performed according to standard methods detailed elsewhere (21, 30).

Cell Surface Labeling and Immunoprecipitation. Approximately 2 × 10^7 cells of each line were labeled with Na^{125}I by lactoperoxidase-catalyzed cell surface iodination. Labeled membrane proteins were then extracted under conditions that disrupt (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl$_2$, 2% NP-40, 1 mM PMSF) or preserve (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl$_2$, 1% digitonin, 1 mM PMSF) the noncovalent associations of the TCR/CD3 complex. Lysates were precleared for 30 min at room temperature (RT) with 2 μl of a mixture of mouse and hamster serum, plus 50 μl of PBS-washed protein G–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Recovered supernatants were then aliquoted and mAb UCHT1 (100 μl tissue culture supernatant) or 145-2C11 (5 μl purified antibody), or rodent serum (1 μl) was added followed by 50 μl protein G–Sepharose (1 h, RT, with gentle rotation). Sepharose beads were then washed three times at RT in their respective lysis buffers, before boiling in reducing sample buffer and analysis by 10% PAGE.

Immunofluorescence and Fluorescence Resonance Energy Transfer. The distribution of surface antigens was determined by direct or indirect immunofluorescence, as described elsewhere (30), using a FACScan analyzer (B-D). List-mode information from 10^4 viable cells was accumulated for each analysis. Surface modulation of the TCR/CD3 complex was performed as described by Meuer et al. (31). We used a FACS to determine energy transfer between T cell surface molecules bound by FITC- and TRITC-labeled mAb, as described in detail by Szollosi et al. (32). Briefly, FITC and TRITC were sequentially excited at 488 and 514 nm, respectively. By using suitable filter combinations, we detected FITC and TRITC emissions at 535 nm (filter bandpath width of 15 nm) and >590 nm, respectively. Compensation parameters were determined from data obtained using single-labeled cells. Results represent the efficiency

---

[1] *Abbreviations used in this paper:* FRET, fluorescence resonance energy transfer; h, human; m, mouse; RT, room temperature; TRITC, tetramethylrhodamine isothiocyanate.
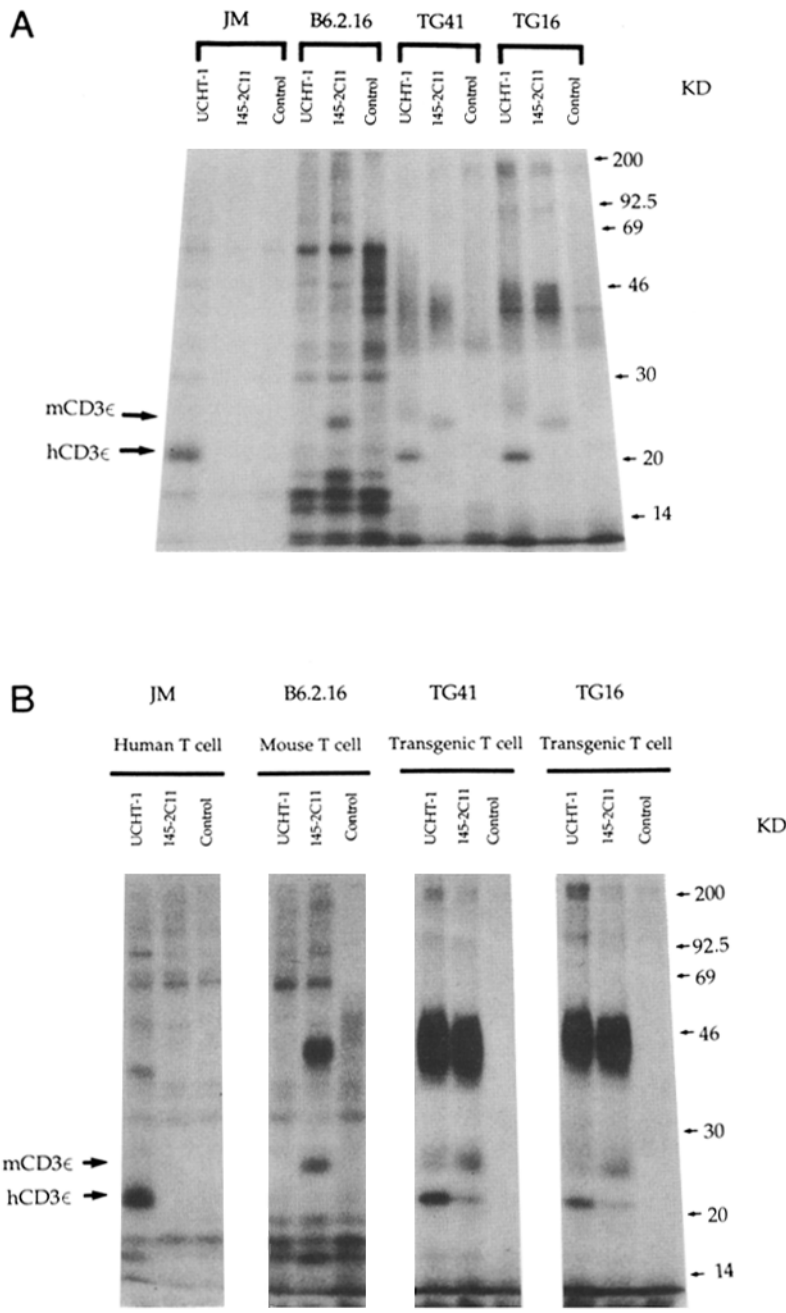
of fluorescence resonance energy transfer (FRET; 32–34), where the efficiency of transfer of (nonradiative) energy from donor to acceptor is determined by measuring fluorochrome acceptor emission as well as the quenching of donor fluorescence emission (radiated photons). The efficiency of transfer is directly related to the average donor-acceptor distance (33), antigens in higher abundancy being preferred as acceptors (32–34).

Results

Analysis of CD3ε Subunit Stoichiometry Using Transgenic Mice. We decided to quantitate the number of CD3ε subunits in the TCR/CD3 complex by taking advantage of the unique coexpression of two distinguishable CD3ε chains on the T cell surface in hCD3ε transgenic mice (21). Five independent strains of transgenic mice were constructed using a cosmid containing the hCD3ε gene and the *cis*-acting elements required for its appropriate expression. The five strains contained from 1 to 90 copies of the hCD3ε gene and were all found to express hCD3ε mRNA in the correct T cell–specific fashion as defined by Northern blot assays (Ollson, et al., manuscript in preparation). T cells derived from independent transgenic strains were submitted to analyses of the subunit associations between the introduced hCD3ε and the endogenous TCR/CD3 components. In particular, we asked whether h- and mCD3ε subunits occur in the same TCR/CD3 complex by using species-specific anti-CD3ε mAb in immunoprecipitation, energy transfer, and surface modulation studies.

Coprecipitation of h- and mCD3ε from Transgenic Mouse T Cells. Initially, we carried out immunoprecipitation studies using surface-labeled cells and mAb specific for either hCD3ε or mCD3ε. ^{125}I-labeled cells were first lysed using detergent conditions that dissociate TCR heterodimer and CD3 subunits. Under these conditions (Fig. 1 *A*), anti-hCD3ε mAb UCHT1 specifically immunoprecipitated a 20-kD polypeptide from T cell lines derived from transgenic mice strains TG16 and TG41 that comigrates with CD3ε from the human T cell line JM. A distinct 25-kD species that comigrates with mCD3ε from nontransgenic littermate T cells was immunoprecipitated by anti-mCD3ε mAb 145-2C11 from a population of transgenic mouse T cells. Thus, the difference in the molecular weights of h- and mCD3 chains allowed us readily to detect their surface coexpression in the transgenic mouse T cells.

We then analyzed the interaction between the two CD3ε subunits and the other endogenous TCR/CD3 components by using aliquots of ^{125}I-labeled cells that were lysed under detergent conditions that preserve TCR/CD3 association. As shown in Fig. 1 *B*, the endogenous 25-kD mCD3ε subunit, as well as the 41–45-kD TCR dimer, were coimmunoprecipitated together with the 20-kD hCD3ε by either anti-mCD3ε or anti-hCD3ε mAb from the surface of transgenic mouse T cells. The specificity of the coprecipitation of h- and mCD3ε was confirmed using human and mouse T cell lines as controls in parallel experiments. A transgene-negative littermate gave very similar results to those of the mouse T cell line (not shown).

A

JM B6.2.16 TG41 TG16

UCHT-1 145-2C11 Control (repeated per group)

KD

← 200
← 92.5
← 69
← 46
← 30
← 20
← 14

mCD3ε →
hCD3ε →

B

JM — Human T cell
B6.2.16 — Mouse T cell
TG41 — Transgenic T cell
TG16 — Transgenic T cell

UCHT-1 145-2C11 Control (per group)

KD

← 200
← 92.5
← 69
← 46
← 30
← 20
← 14

mCD3ε →
hCD3ε →

Figure 1. Coprecipitation of h- and mCD3ε in T cells from transgenic mice. The indicated human, mouse, or hCD3ε transgenic mice T cell lines were surface iodinated and aliquots lysed under conditions that either disrupt (*A*) or preserve (*B*) the noncovalent associations of the TCR/CD3 complex. Lysates were submitted to precipitation with antibodies specific for either hCD3ε (UCHT1) or mCD3ε (145-2C11), or with control rodent sera, followed by 10% PAGE. The autoradiogram was exposed for 48 h. Bold arrows indicate the localization of h- and mCD3ε in the gel. The precipitation pattern of the mouse T cell using mAb 145-2C11, specific for mCD3ε, shows that, under the conditions used, mCD3γ (which has a similar size to hCD3ε) is very poorly labeled and is not detected. In the corresponding immunoprecipitation with the mAb specific for human CD3ε, some of the 25–26-kD region may contain mouse CD3δ in addition to mouse CD3ε.

The coprecipitation of h- and mCD3ε in the digitonin lysates (Fig. 1 *B*) shows that a proportion of TCR/CD3 complexes contains at least two CD3ε subunits. The lack of such coprecipitation in the NP40 lysates (Fig. 1 *A*) implies that the predominant association of h- and mCD3ε is not due to strong, direct ε-ε interactions.

Fluorescence Resonance Energy Transfer between h- and mCD3ε Subunits. We examined further the physical relationship between the human and endogenous CD3ε chains by using a different experimental method: flow cytometric analysis and FRET. FRET has been used successfully to determine intra- and intermolecular distances at the 2- to 10-nm level in single

cells (32–34). In our study, we used mAbs against mCD3ε and hCD3ε that were suitably labeled with either FITC or TRITC. FITC is excited at ∼488 nm and can serve as non-radiative energy donor for a nearby (<10-nm distant) TRITC acceptor molecule. The relative spatial distribution of the binding sites for FITC-anti-mCD3ε and TRITC-anti-hCD3ε was investigated in mouse transgenic T cells. Cells labeled with both conjugated mAb were analyzed to measure the efficiency of energy transfer. The fluorescence distribution found implies energy transfer (Table 1) and indicates that anti-mCD3ε and anti-hCD3ε mAbs and their respective binding sites are in close proximity (<10 nm). The reciprocal donor-

Table 1. *Fluorescence Resonance Energy Transfer between Donor-Acceptor Pairs of mAb*

| T cells | Donor (FITC) | Acceptor (TRITC) | T^a | Efficiency (mean percent) | | Transfer |
				TG16	TG41	
CD3ε transgenic mice:						
	mCD3ε	hCD3ε	4°C	11.3, 10.7	12.3, 10.2	Yes
	hCD3ε	mCD3ε	4°C	12.9, 11.2	10.6, 11.0	Yes
	hCD3ε	mTCRβ	4°C	13.7, 13.4	ND	Yes
	mCD3ε	mTCRβ	4°C	13.8, 14.0	ND	Yes
	mCD3ε	CD4 + CD8	4°C	0.4, 0.6	0.7, 0.3	No
	hCD3ε	CD4 + CD8	4°C	0.3, 0.5	0.9, 0.2	No
	mCD3ε	CD4 + CD8	37°C	8.4, 7.3	9.6, 7.5	Yes
Nontransgenic littermate:						
	mCD3ε	hCD3ε	4°C	0.3, 0.1	0.4, 0.2	No

Spleen T cells from either CD3ε transgenic or control littermate mice were enriched using nylon wool columns (21). The frequency distribution of energy transfer efficiency between fluoresceinated (FITC) and rhodaminated (TRITC) mAbs specific for the indicated antigens on the surface of transgenic T cells was measured by flow cytometry. The cells were incubated with the antibodies for 60 min at the indicated temperatures (T^a). Mean efficiency of transfer is displayed for T cells from two individual mice for either the TG16 or the TG41 strains, and control littermates. The interpretation of energy transfer results, where "Yes" corresponds to transfer efficiency of ≥2%, is indicated in the Transfer column.

acceptor combination, FITC-anti-hCD3ε and TRITC-anti-mCD3ε, yielded similar results. The m- and hCD3ε-specific pair of mAbs used did not induce energy transfer in nontransgenic T cells.

When similar experiments were carried out using donor/acceptor antibody pairs specific for either h- or mCD3ε and mTCR-β, values of efficiency of FRET were in agreement with the hCD3ε/mCD3ε pair results (Table 1). This indicates that both the h- and mCD3ε chains and the TCR-β are in close proximity to each other.

Neither TRITC-anti-CD4 nor TRITC-anti-CD8 mAb, which bind to antigens that are about fourfold more abundant on the T cell surface, acted as acceptors in combination with a FITC-anti-CD3ε mAb donor (Table 1). It is worthy to note that energy transfer between anti-CD4 and anti-CD3 fluorochrome-tagged mAb can take place at 21–37°C after microaggregation of surface molecules, but does not occur at 4°C (34; Table 1). This rules our artifactual energy transfer occurring between distant molecules under our working conditions (32–34). The FRET results show that the hCD3ε-mTCR/CD3 association first detected by immunoprecipitation occurs in all transgenic T cells, and not just in a subpopulation.

Partial Comodulation Implies Random Association of h- and mCD3ε. The above results imply that the TCR/CD3 complex contains at least two CD3ε subunits. If hCD3ε can structurally substitute for mCD3ε, the existence of TCR/CD3 complexes containing only either hCD3ε or mCD3ε subunit pairs should be detectable. We examined this possibility by modulation of the surface expression of either hCD3ε or mCD3ε subunits and subsequent immunofluorescence and flow cytometry analysis of the reciprocal chain with specific mAb (Fig. 2). Although anti-mCD3ε mAb removed most

(~95%) of surface mCD3ε, the expression of hCD3ε was only reduced by ~60% as compared with modulation with anti-hCD3ε mAb. Similarly, anti-hCD3ε mAb removed only a portion (~50%) of mTCR/CD3 complexes in every cell after virtually complete modulation of hCD3ε antigen. In contrast, >95% of both hCD3ε and mCD3ε comodulated with TCR-α/β (Fig. 2), indicating that both types of CD3ε subunit are integral antigen receptor components. The specificity of the comodulation was confirmed using Thy-1 antigen as control.

These data are consistent with a TCR/CD3 complex that randomly assembles hCD3ε and mCD3ε subunits within a single receptor, the simplest interpretation being that each complex contains two CD3ε chains. Given random usage of either h- or mCD3ε in the transgenic mice, a ratio of 1:2:1 of the three species of TCR (comprising m/m-, h/m-, and h/hCD3ε, respectively) would result. Therefore, since mε is represented twice in the mε/mε TCR/CD3 species, half of the surface mCD3ε would be found in hε/mε-type receptors, and half in mε/mε complexes, when expressed in molar amounts. In this case, antibodies against hCD3ε would be expected to comodulate half of the mCD3ε chains from the cell surface, which is close to the experimental result. For hypothetical receptor complexes with three (or more) CD3ε chains, HCD3ε-specific antibodies should comodulate 75% (or more) of the surface mCD3ε, which is not observed experimentally. The antigen comodulation data, therefore, strongly suggest a stoichiometry of two CD3ε subunits per TCR.

Analysis of T Cell Development in hCD3ε Transgenic Mice. We were concerned to demonstrate that the chimeric antigen receptors behaved normally in the transgenic mice, and there-

Surface antigen modulated
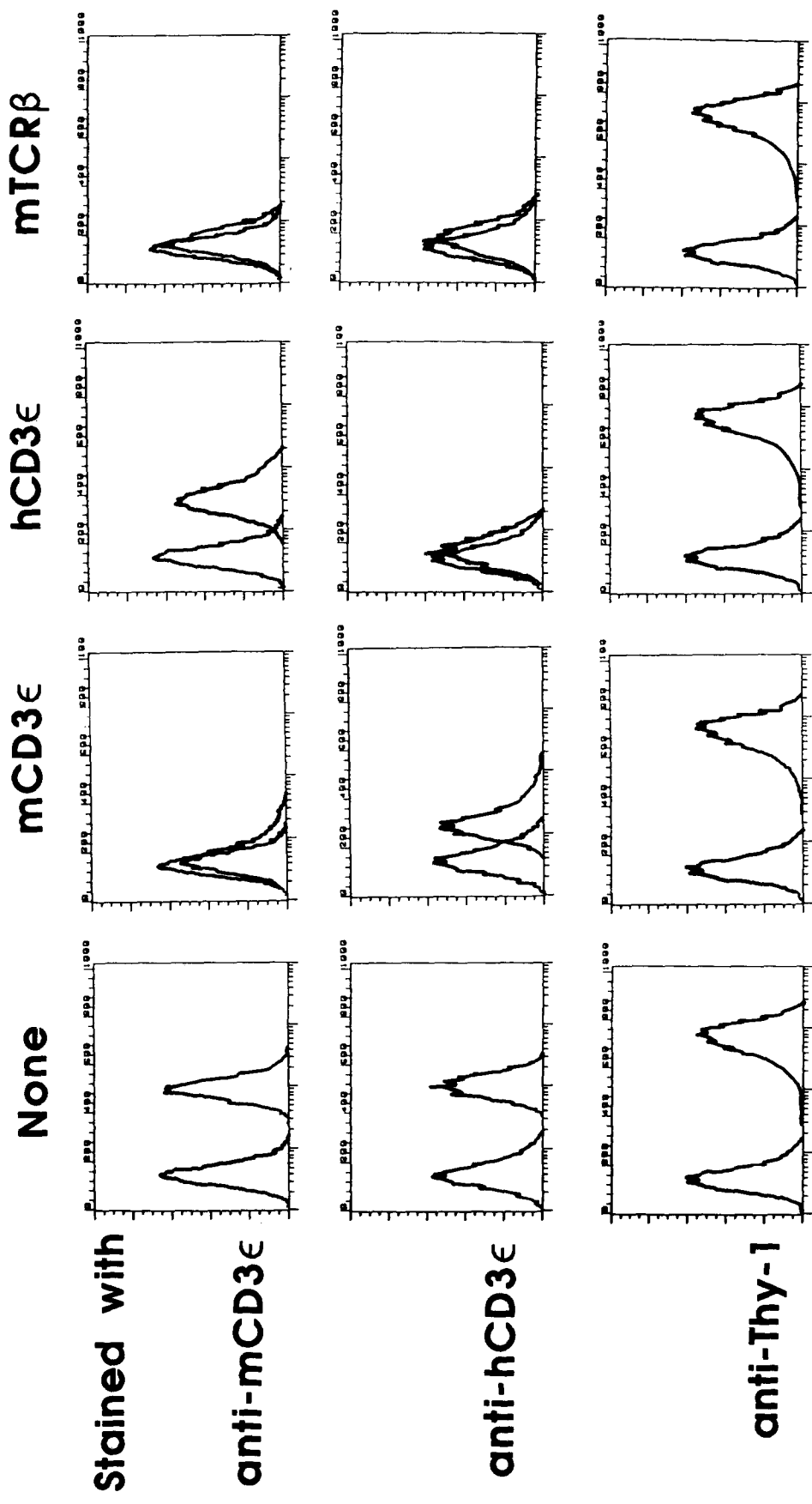
LOG GREEN FLUORESCENCE

Figure 2. Surface modulation of TCR/CD3 complex. T cells from TG16 mice were incubated overnight either in complete culture media alone (*None*) or supplemented with antibodies specific for the indicated antigens. To measure the comodulation of TCR/CD3 subunits, cultured cells were washed and stained with antibodies specific for either mCD3-ε, hCD3ε, or an unrelated surface antigen, Thy-1. In each group, the antigen submitted to modulation was analyzed by indirect immunofluorescence, whereas other antigens were studied by direct immunofluorescence. Results are displayed as histograms of fluorescence distribution in a logarithmic scale. Left histograms in each panel represent background staining using an irrelevant hCD2 mAb, whereas right histograms represent staining with the specific mAb.

11   de la Hera et al.
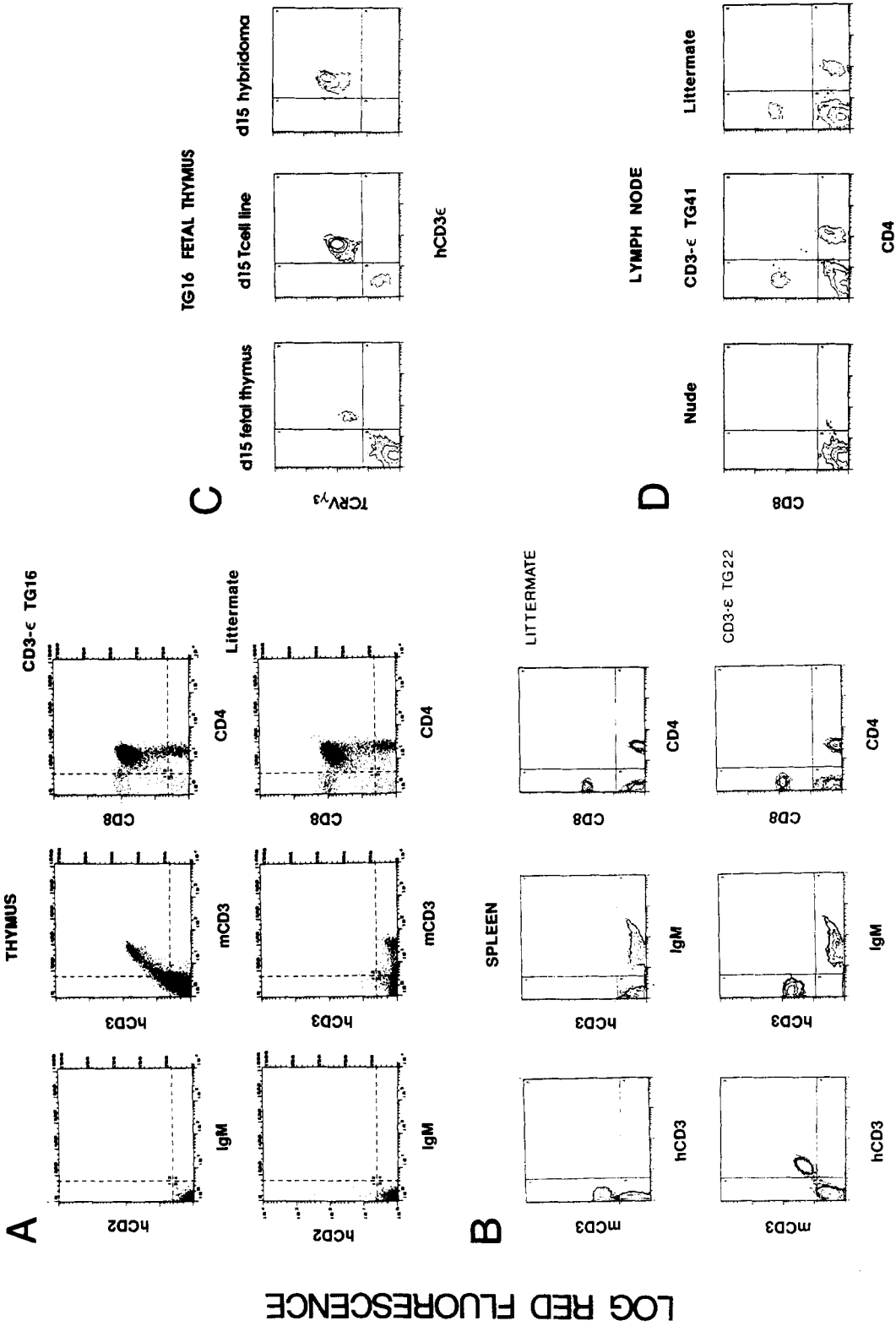
LOG GREEN FLUORESCENCE

Figure 3. Two-color immunofluorescence and FACS analysis for coordinate expression of T cell differentiation antigens in hCD3ε transgenic mouse strains. Cells were stained with antibodies specific for the indicated surface antigens. Antibodies were either directly labeled with FITC (green fluorescence axis) or biotin conjugated followed by a second step of streptavidin-PE (red fluorescence axis). Analyses were performed in a FACScan analyzer equipped with a four-decade log amplifier. 10⁴ viable cells were accumulated in each plot. Background values, obtained using irrelevant antibodies, are indicated by lines in each panel.

fore, that their function, and hence structure, reflected that found in normal mice. Along these lines, we compared by immunofluorescence and flow cytometry the thymocyte and peripheral lymphoid populations in transgenic and control mice to study the putative effects of transgene expression upon T cell development in vivo. First, we studied the effect of the transgene upon the level of CD3 expression. As shown in Fig. 3 A, subpopulations (49, 35, and 16%, respectively) of transgenic thymocytes bear either no, low, or high amounts of hCD3ε chains, whereas virtually all spleen and lymph node T cells (i.e., mCD3$^+$, Ig$^-$) selectively express high levels of hCD3ε (Fig. 3 B). It is worth noting that, both in the thymus and peripheral lymphoid organs, the transgenic and endogenous CD3ε chains were coexpressed in similar amounts on the surface of every T cell, each in roughly half of the amount of mCD3ε found in nontransgenic littermates (Figs. 2 and 3). Thus, the total (h + m) amount of CD3ε chains expressed in T cells from the transgenic mice approximately equals that found in normal mice.

Second, we studied the association of hCD3ε with α/β or γ/δ types of TCR. The majority of T cells in the thymus and periphery express TCR-α/β associated with hCD3ε (Fig. 2), whereas the T cells that arise first in ontogeny, occurring in low numbers at day 15 in fetal thymus (25), instead bear TCR-γ/δ associated with hCD3ε, as found after analysis of fresh thymocytes and confirmed using T cell lines and hybridomas derived from that cell population (Fig. 3 C). Thus, both T cell lineages express physiological levels of TCR, hCD3ε, and mCD3ε during ontogeny and in the periphery.

Third, we analyzed the distribution of CD4 and CD8, molecules implicated as TCR/CD3 coreceptors in the processes of positive and negative selection of T cells (35). The absolute number of cells found in lymphoid organs, and the proportions of cells in T cell subsets defined by the expression of CD4 and CD8 surface markers, were all within the range found in nontransgenic littermates (Fig. 3 A, B, and D).

Finally, we monitored the effect upon in vivo selection of TCR repertoires of the chimeric TCR/CD3 expressed in the transgenic mice T cells (Table 2). Bill et al. (26) have shown that DBA/2 and [B6 × DBA/2]F$_1$ (BDF$_1$) mice have significantly reduced numbers of V$_{β11}$ cells, whereas C57BL/6 (B6) T cells, which do not express E$_α$E$_β$ products necessary for deletion of V$_{β11}$-bearing T cells, do not have deletions of T cells expressing V$_{β11}$ (26). The founder mice for our hCD3ε transgenic strains were BDF$_2$, the initial backcross was with B6 mice, and the positive offspring was subsequently subjected to brother/sister mating. Therefore, the homozygous transgenic individual analyzed can either bear or lack expression of I-E gene products depending on the segregation of the MHC haplotype. We found that low frequencies of V$_{β11}$-bearing T cells indeed correlate with the expression of I-E in the CD3ε transgenic mice analyzed (Table 2). The frequencies of T cells bearing either V$_{β6}$ or V$_{β8}$ domains are not significantly modified in the same mice, indicating the specificity of the deletion occurring in the transgenic mice (Table 2). We conclude that association of hCD3ε with the mouse TCR heterodimer and CD3 complex does not perturb negative selection during T cell development. In summary, after the analysis of >100 individuals from five different transgenic lines from day 14 of fetal age to 1 yr old, it is clear that the surface expression of hCD3ε and the subsequent generation of h/m chimeric antigen receptors does not disrupt normal T cell development in our homozygotic transgenic mice.

hCD3ε-containing TCR/CD3 Complexes Are Functional in Assays In Vitro. We also asked whether the presence of hybrid hCD3ε-mTCR/CD3 receptors in transgenic T cells affects the function of TCR/CD3 complex as measured by proliferation and cytotoxicity assays: spleen cells of the transgenic mice showed a strong proliferative response to hCD3ε-specific mAb (Table 3). The response was similar to that obtained using either mTCR-β- or mCD3ε-specific mAb in spleen cells from both transgenic and nontransgenic mice. We examined whether anti-CD3-stimulated cells expanded in IL-2 mediate TCR/CD3-dependent cytolytic activity (Table 4). Target cells coated with anti-hCD3ε, as well as mCD3ε- or βTCR-specific

Table 2. *Expression of VβTCR Domains in T cells*

Strain	Cells stained with mAb specific for:			
	V$_{β6}$	V$_{β8}$	V$_{β11}$	Surface I-E expression
C57BL/6	8.9 ± 0.2	19.8 ± 0.6	4.8 ± 0.7	Negative
DBA/2	11.7 ± 0.3	24.1 ± 1.2	1.5 ± 0.2	Positive
[B6 × DBA/2]F$_1$	10.9 ± 0.5	21.1 ± 0.7	1.3 ± 0.1	Positive
TG16 (I-E$^+$)	10.7 ± 0.6	22.8 ± 0.3	1.4 ± 0.3	Positive
TG16 (I-E$^-$)	9.8 ± 0.3	19.2 ± 0.4	4.7 ± 0.6	Negative

Spleen cells were enriched for T cells using nylon wool columns (21), and 5 × 10^5 cells in each group were incubated with the indicated biotin-conjugated Vβ-specific mAb followed by streptavidin-pe and FITC-labeled anti-mCD3. The number of cells stained by Vβ-specific mAb is represented as a mean percentage ± SE of total CD3$^+$ cells of results obtained for individual mice, with 6–10 mice in each strain analyzed. Similar results were obtained with TG41 strain. The level of expression of I-E^d molecules was measured in unfractionated spleen cells using FITC-conjugated 14.4.4 mAb and flow cytometry using an irrelevant CD2-specific mAb as negative control.

Table 3. *Proliferation of Cells Stimulated with TCR/CD3-specific Antibodies*

Cell source	Proliferation of cells stimulated with:			
	hCD2	HCD3ε	mCD3ε	mTCR-β
	cpm			
TG16	1,583	37,051	43,808	38,944
Nontransgenic littermate	1,897	1,766	46,122	39,577
Human peripheral blood	1,193	53,873	1,087	1,154

10^5 cells mice spleen or human peripheral blood cells were stimulated with the indicated antibodies (0.5 μg/ml) and cultured for 4 d in 200 μl complete medium (21, 30). [^{3}H]Methyl-thymidine was added to the wells 8 h before the harvesting. Results show the mean thymidine incorporation of triplicate wells in one experiment representative of five performed.

Table 4. *hCD3ε-containing Antigen Receptor Complexes Are Functional in Cytoxicity Assays*

Effector T cell origin	Percentage of specific lysis of U937 cells coated with:			
	hCD2	hCD3ε	mCD3ε	mTCR-β
TG16	6 ± 1	86 ± 5	82 ± 6	79 ± 4
Nontransgenic littermate	7 ± 4	5 ± 2	91 ± 4	81 ± 6
Human peripheral blood	8 ± 1	91 ± 5	7 ± 2	7 ± 1

5×10^4 mouse spleen or human peripheral blood T cells that had been polyclonally stimulated with anti-CD3 antibodies and cultured for 10 d in the presence of 25 U/ml rII-2 (21) were used as effector cells. They were incubated for 4 h at different E/T ratios with ^{51}Cr-labeled U937 cells precoated with the indicated antibodies (30). Results show the mean ± SE ^{51}Cr-specific release at 10:1 E/T ratio in one experiment representative of four performed.

mAb, but not irrelevant antibodies, were efficiently killed by transgenic T cells. Both the proliferative and cytotoxic responses promoted by anti-hCD3ε mAb were specific since they were not detected using nontransgenic T cells. We conclude therefore that the hybrid hCD3ε-mTCR/CD3 receptors are functionally competent.

Discussion

Immunoprecipitation and FRET analyses in T cells from transgenic mice that carry the hCD3ε gene have shown that the hCD3ε chain is able to replace mCD3ε subunits in the assembled TCR/CD3 complex, and that transgenic TCRs can contain both h- and mCD3ε. Antigen modulation studies demonstrated that there are two CD3ε chains per antigen receptor and defined three receptor species, hε/hε, hε/mε, and mε/mε, in a ratio of 1:2:1, respectively, suggesting a stochastic use of human or mouse CD3ε during TCR/CD3 assembly. The chimeric antigen receptors support normal T cell development and selection of repertoires in vivo and are

able to transduce activation signals in vitro. These findings imply that hCD3ε can both structurally and functionally substitute for mCD3ε in the transgenic mouse strains studied and show that the TCR/CD3 complex contains two CD3ε subunits.

Previously, the subunit stoichiometry of the TCR/CD3 complex was considered to be either αβγδεζ₂ or αβγδεζη (18–20). However, conclusive data supporting such a representation are lacking. The demonstration that there are two CD3ε chains in a functional antigen receptor, together with the data of other workers, allows us to present a minimal model for the TCR/CD3 complex (Fig. 4). Several biochemical studies of partial receptor complexes have demonstrated four preferential subunit pairs: α/β, ζ₂, γ/ε, and δ/ε (but not CD3γ/δ; 36–38), and the model is built up from these pairs. The TCR-α/β and ζ₂ dimers can associate with each other in the absence of CD3 (37), suggesting that the most important structural interaction of ζ₂ is with the TCR heterodimer (Fig. 4). Chemical crosslinking experiments indicate
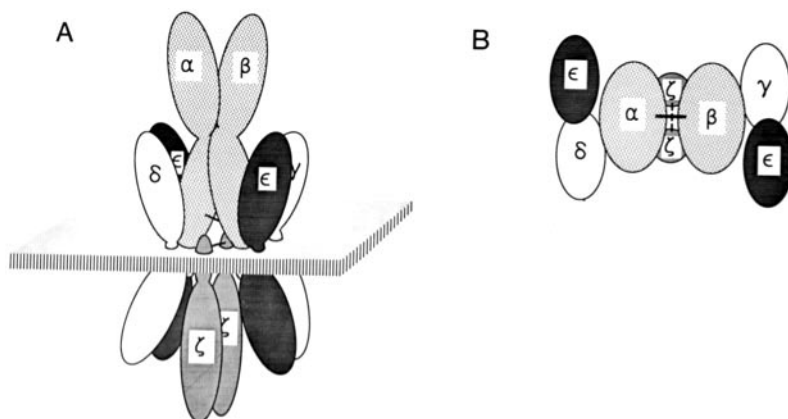

Figure 4. Minimal model of the TCR/CD3 complex. (*A*) Cross-section through the T cell membrane showing the stoichiometry and associations of TCR/CD3 subunits. "Like" subunits (TCR-α and -β; CD3-γ and -δ) are shaded identically to emphasize structural symmetry. (*B*) Plan view of the receptor from outside the T cell. Disulfide bonds are shown as solid (TCR-α/β) or dotted (ζ-ζ) lines.

the proximity of CD3γ to TCR-β (38, 39) and of CD3ε to both TCR-α and -β (38). This is consistent with a CD3γ/ε heterodimer lying adjacent to the TCR β chain of the receptor, and a CD3δ/ε pair flanking TCR-α (Fig. 4).

The model predicts that CD3δ contacts TCR-α and not -β, and further suggests that specificity of contact between CD3 chains and TCR-α or -β must reside in the CD3γ and -δ subunits, since CD3ε associates with both chains of the TCR heterodimer. However, such specificity must also allow association of CD3 with TCR-γ/δ chains. We have shown expression of the human CD3ε chain on γ/δ T cells from ε-transgenic mice, and, although we have not investigated the structure of the TCR-γ/δ/CD3 complex in detail, we feel it is likely that the overall organization will be similar to that of TCR-γ/β/CD3. Considering the demonstration of a functional TCR-δ/β/CD3 complex (40), the most parsimonious interpretation would be that TCR-δ and -γ substitute for TCR α and β chains, respectively, in the TCR-γ/δ/CD3 complex.

The minimal model for the TCR has a stoichiometry of $\alpha\beta\gamma\delta\epsilon_2\zeta_2$. While more complex stoichiometries are conceivable, particularly since at present the minimum representation of chains other than CD3ε and -ζ is not clear, certain of these structures are improbable. For example, a TCR/CD3 complex containing two copies of each CD3 subunit ($\gamma_2\delta_2\epsilon_2$) is unlikely for the following reasons. Since CD3γ/δ dimers are not found (36–38), a CD3$\gamma_2\delta_2\epsilon_2$ complex would have to be grouped as trimers, either $(\gamma\delta\epsilon)_2$ or $\gamma_2\epsilon$ plus $\delta_2\epsilon$. CD3γ/δ/ε subcomplexes have not been documented: although anti-CD3γ sera coprecipitate CD3ε, they do not detect CD3δ. Similarly, anti-CD3δ sera do not coprecipitate CD3γ (38). Previous immunoprecipitation data suggesting a CD3γ/δ/ε trimer (37, 41) can be seen as the simultaneous precipitation of γ/ε and δ/ε species by the CD3ε-specific antibodies used. Therefore, an antigen receptor with a representation of CD3$\gamma_2\delta_2\epsilon_2$ would involve the formation of $\gamma_2\epsilon$ and $\delta_2\epsilon$ trimers. This would require CD3ε to possess duplicated association sites for both CD3γ and -δ chains, or to bind one subunit of a γ/γ (or δ/δ) dimer. Although possible, these arrangements seem artificially complex compared with the simple γ/ε and δ/ε dimers proposed in Fig. 4, where CD3γ and -δ associate in a mutually exclusive manner with CD3ε, possibly competing for the same binding site on the CD3ε

protein. This is consistent with the high degree of relatedness of CD3γ and -δ: one or both of the two conserved sequence motifs found in all CD3γ and -δ proteins from three species (42) may be involved in association with CD3ε. However, additional experiments will be required to provide direct evidence for the stoichiometry of other receptor components and, in particular, to examine the intriguing possibility that the antigen binding subunits could form a tetramer ($\alpha_2\beta_2$), like Igs, rather than a dimer.

There are a number of consequences of a TCR with two CD3ε subunits. (a) The majority of CD3-specific mAbs recognize epitopes conferred by CD3ε (21, 43) and therefore have the potential to form strings of linked TCR/CD3 complexes on the cell surface. This crosslinking function may be the critical mitogenic property of CD3-specific mAbs, since antibodies monovalent for CD3 are nonstimulatory in the absence of secondary crosslinking (44). (b) CD3ε-specific mAbs may also crosslink the two CD3ε chains within a single TCR, assuming no steric hindrance from other subunits. It is conceivable that inter-receptor and intra-receptor crosslinking might lead to qualitatively or quantitatively different intracellular signals. (c) If a TCR/CD3 complex contains a single α/β heterodimer, TCR-α/β-specific mAbs would have only a limited capacity for receptor crosslinking. This contrasts with CD3ε-specific mAbs, and might account for differences in the abilities of the two types of mAbs to stimulate T cells during T cell selection in thymus organ culture (45).

Interspecies polymorphism and complementation of TCR/CD3 subunits allowed the definition of CD3ε stoichiometry in transgenic mouse T cells. The transgenic approach to the analysis of structure/function relationships of the TCR offers several advantages over in vitro systems. It can facilitate the examination of the structure of the receptor: (a) at early developmental stages, where there is limited knowledge of TCR/CD3 subunit expression, assembly, and function; (b) during negative and positive selection processes; and (c) in the several lineages and subsets derived from T cell precursors, since the different cell populations (or lines) characterized in each strain have the same defined genetic background and do not need to be transformed. Transgenic mice may prove to be of general value as a technique for the analysis of complex receptors.

Address correspondence to Antonio de la Hera, Basel Institute for Immunology, Grenzacherstrasse 487, Basel CH-4058, Switzerland.

References

1. Clevers, H., B. Alarcon, T. Wileman, and C. Terhorst. 1988. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu. Rev. Immunol.* 6:629.

2. Baniyash, M., P. Garcia-Morales, J.S. Bonifacino, L.E. Samelson, and R.D. Klausner. 1988. Disulfide linkage of the ζ and η chains of the T cell receptor. *J. Biol. Chem.* 263:9874.

3. Sussman, J.J., J.S. Bonifacino, J. Lippincott-Schwartz, A.M. Weissman, T. Saito, R.D. Klausner, and J.D. Ashwell. 1988. Failure to synthesize the T cell CD3-ζ chain: structure and function of a partial T cell receptor complex. *Cell.* 52:85.

4. Weissman, A.M., S.J. Frank, D.G. Orloff, M. Mercep, J.D. Ashwell, and R.D. Klausner. 1989. Role of the zeta chain in the expression of the T cell antigen receptor: genetic reconstitution studies. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3651.

5. Geisler, C., J. Kuhlmann, and B. Rubin. 1989. Assembly, intracellular processing, and expression at the cell surface of the human αβ T cell receptor/CD3 complex. *J. Immunol.* 143:4069.

6. Sancho, J., T. Chatila, R.C.K. Wong, C. Hall, R. Blumberg, B. Alarcon, R.S. Geha, and C. Terhorst. 1989. T-cell antigen receptor (TCR)-α/β heterodimer formation is a prerequisite for association of CD3-ζ₂ into functionally competent TCR-CD3 complexes. *J. Biol. Chem.* 264:20760.

7. Cantrell, D.A., A.A. Davies, and M.J. Crumpton. 1985. Activators of protein kinase C down-regulate and phosphorylate the T3/T-cell antigen receptor complex of human T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 82:8158.

8. Alexander, D.R., and D.A. Cantrell. 1989. Kinases and phosphatases in T-cell activation. *Immunol. Today.* 10:200.

9. Van Wauwe, J.P., J.R. De May, and J.G. Goossens. 1980. OKT3: a monoclonal anti-human T lymphocyte antibody with potent mitogenic properties. *J. Immunol.* 124:2708.

10. Mercep, M., J.S. Bonifacino, P. Garcia-Morales, L.E. Samelson, R.D. Klausner, and J.D. Ashwell. 1988. T cell CD3-ζη heterodimer expression and coupling to phosphoinositide hydrolysis. *Science (Wash. DC).* 242:571.

11. Mercep, M., A.M. Weissman, S.J. Frank, R.D. Klausner, and J.D. Ashwell. 1989. Activation-driven programmed cell death and T cell receptor ζη expression. *Science (Wash. DC).* 246:1162.

12. Williams, A.F., and A.N. Barclay. 1988. The immunoglobulin superfamily-domains for cell surface recognition. *Annu. Rev. Immunol.* 6:381.

13. Tunnacliffe, A., C. Olsson, L. Buluwela, and T.H. Rabbitts. 1988. Organization of the human CD3 locus on chromosome 11. *Eur. J. Immunol.* 18:1639.

14. Evans, G.A., K.A. Lewis, and G.M. Lawless. 1988. Molecular organization of the human CD3 gene family on chromosome 11q23. *Immunogenetics.* 28:365.

15. Letourneur, F., M.-G. Mattei, and B. Malissen. 1989. The mouse CD3-γ, -δ, and -ε genes reside within 50 kilobases on chromosome 9, whereas CD3-ζ maps to chromosome 1, band H. *Immunogenetics.* 29:265.

16. Weissman, A.M., D. Hou, D.G. Orloff, and W.S. Modi, H. Seuanez, S.J. O'Brien, and R.D. Klausner. 1988. Molecular cloning and chromosomal localization of the human T-cell receptor ζ chain: distinction from the molecular CD3 complex. *Proc. Natl. Acad. Sci. USA.* 85:9709.

17. Jin, Y.-J., L.K. Clayton, F.D. Howard, S. Koyasu, M. Sieh, R. Steinbrich, G.E. Tarr, and E.L. Reinherz. 1990. Molecular cloning of the CD3η subunit identifies a CD3ζ-related product in thymus-derived cells. *Proc. Natl. Acad. Sci. USA.* 87:3319.

18. Minami, Y., A.M. Weissman, L.E. Samelson, and R.D. Klausner. 1987. Building a multichain receptor: synthesis, degradation, and assembly of the T-cell antigen receptor. *Proc. Natl. Acad. Sci. USA.* 84:2688.

19. Meuer, S.O., O. Acuto, R.E. Hussey, J.C. Hodgdon, K.A. Fitzgerald, S.F. Schlossman, and E.L. Reinherz. 1983. Evidence for the T3-associated 90K heterodimer as the T-cell antigen receptor. *Nature (Lond.).* 303:808.

20. Krissansen, G.W., W. Verbi, N.F. Totty, and M.J. Crumpton. 1987. Purification and identification by amino acid sequence analysis of the subunits of the CD3 antigen of human tonsil T-lymphocytes. *Mol. Immunol.* 24:1069.

21. Tunnacliffe, A., C. Olsson, and A. de la Hera. 1989. The majority of human CD3 epitopes are conferred by the epsilon chain. *Int. Immunol.* 1:546.

22. Beverley, P.C.L., and R.E. Callard. 1981. Distinctive functional characteristics of human "T" lymphocytes defined by E rosetting or a monoclonal anti-T cell antibody. *Eur. J. Immunol.* 11:329.

23. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84:1374.

24. Kubo, R.T., B. Born, J.W. Kappler, P. Marrack, and M. Pigeon. 1988. Characterization of a monoclonal antibody which detects all mouse αβ T cell receptors. *J. Immunol.* 142:2736.

25. Havran, W., and J.P. Allison. 1988. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature (Lond.).* 335:443.

26. Bill, J., O. Kanagawa, D.L. Woodland, and E. Palmer. 1989. The MHC molecule I-E is necessary but not sufficient for the clonal deletion of Vβ₁₁-bearing T cells. *J. Exp. Med.* 169:1405.

27. Kanagawa, O., E. Palmer, and J. Bill. 1989. A T-cell receptor Vβ domain that imparts reactivity to the Mlsᵃ antigen. *Cell. Immunol.* 119:412.

28. Staerz, U., H.G. Ramensee, J.D. Benedetto, and M.J. Bevan. 1985. Characterization of a murine monoclonal antibody specific for an allotypic determinant on the T-cell antigen receptor. *J. Immunol.* 134:3994.

29. Forni, L., and S. de Petris. 1984. Use of fluorescent antibodies in the study of lymphoid cell membrane molecules. *Methods Enzymol.* 108:413.

30. de la Hera, A., M.L. Toribio, and C. Martinez-A. 1989. Deliniation of human thymocytes with or without functional potential by CD1-specific antibodies. *Int. Immunol.* 1:496.

31. Meuer, S.C., K.A. Fitzgerald, R.E. Hussey, J.C. Hodgdon, S.F. Schlossman, and E.L. Reinherz. 1983. Clonotypic structures involved in antigen-specific human T cell function. *J. Exp. Med.* 157:705.

32. Szollosi, J., L. Matyos, L. Tron, M. Balazs, I. Ember, M.J. Fulwyler, and S. Damjanovich. 1987. Flow cytometric measurements of fluorescence energy transfer using simple laser excitation. *Cytometry.* 8:120.

33. Szollosi, J., S. Damjanovich, C.K. Goldman, M.J. Fulwyler, S.D. Aszalos, G. Goldstein, P. Rao, M.A. Talle, and T.A. Waldmann. 1987. Flow cytometric resonance energy transfer measurements support the association of a 95Kda peptide termed T27 with the 55Kda Tac peptide. *Proc. Natl. Acad. Sci. USA.* 84:7246.

34. Mittler, R.S., S.J. Goldman, G.L. Spitalny, and S.J. Burakoff. 1989. T-cell receptor-CD4 physical association in a murine T-cell hybridoma. Induction by antigen receptor ligation. *Proc. Natl. Acad. Sci. USA.* 86:8531.

35. von Boehmer, H., H.S. Teh, and P. Kisielow. 1988. The thymus

selects the useful, neglects the useless and destroys the harmful. *Immunol. Today.* 10:57.

36. Berkhout, B., B. Alarcon, and C. Terhorst. 1988. Transfection of genes encoding the T cell receptor-associated CD3 complex into COS cells results in assembly of the macromolecular structure. *J. Biol. Chem.* 263:8528.

37. Bonifacino, J.S., C. Chen, J. Lippincott-Schwartz, J.D. Ashwell, and R.D. Klausner. 1988. Subunit interactions within the T-cell antigen receptor: clues from the study of partial complexes. *Proc. Natl. Acad. Sci. USA.* 85:6929.

38. Koning, F., W.L. Maloy, and J.E. Coligan. 1990. The implications of subunit interactions for the structure of the T cell receptor-CD3 complex. *Eur. J. Immunol.* 20:299.

39. Brenner, M.B., I.S. Trowbridge, and J.L. Strominger. 1985. Cross-linking of human T cell receptor proteins: association between the T cell idiotype β subunit and the T3 glycoprotein heavy subunit. *Cell.* 40:183.

40. Hochstenbach, F., and M.B. Brenner. 1989. T-cell receptor δ-chain can substitute for α to form a βδ heterodimer. *Nature (Lond.).* 340:562.

41. Alarcon, B., B. Berkhout, J. Breitmeyer, and C. Terhorst. 1988. Assembly of the human T cell receptor-CD3 complex takes place in the endoplasmic reticulum and involves intermediary complexes between the CD3-γ·δ·ε core and single T cell receptor α or β chains. *J. Biol. Chem.* 263:2953.

42. Hein, W.R., and A. Tunnacliffe. 1990. Characterization of the CD3γ and δ invariant subunits of the sheep T cell antigen receptor. *Eur. J. Immunol.* 20:1505.

43. Transy, C., P.E. Moingeon, B. Marshall, C. Stebbins, and E.L. Reinherz. 1989. Most anti-human CD3 monoclonal antibodies are directed to the CD3 ε subunit. *Eur. J. Immunol.* 19:947.

44. Roosnek, E., and A. Lanzavecchia. 1989. Triggering T cells by otherwise inert hybrid anti-CD3/anti-tumor antibodies requires encounter with the specific target cell. *J. Exp. Med.* 170:297.

45. Finkel, T.H., P. Marrack, J.W. Kappler, R.T. Kubo, and J.C. Cambier. 1989. αβT cell receptor and CD3 transduce different signals in immature T cells. *J. Immunol.* 142:3006.