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### Permalink

<https://escholarship.org/uc/item/8vv91677>

### Journal

Journal of Experimental Medicine, 173(5)

### ISSN

0022-1007

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### Publication Date

1991-05-01

### DOI

10.1084/jem.173.5.1247

Peer reviewed

# Regions of the T Cell Receptor $\alpha$ and $\beta$ Chains that Are Responsible for Interactions with CD3

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## Summary

The T cell antigen receptor consists of the Ti  $\alpha/\beta$  heterodimer which recognizes antigen, and the associated CD3 chains, thought to be involved in signal transduction. To understand the nature of the interaction between Ti and CD3, chimeric molecules which included the COOH-terminal segments of Ti  $\alpha$  or  $\beta$  linked to the extracellular segment of CD3, were transfected into a mutant T cell deficient in Ti $\beta$  chain expression and cell surface CD3. Both chimeric chains were required to express the chimeric Ti and to restore CD3 surface expression. CD3/Ti and CD3 cointernalized and coimmunoprecipitated. Stimulation of the chimeric receptor induced transmembrane signaling events and cell activation. These results demonstrate that the Ti  $\alpha$  and  $\beta$  COOH termini containing the transmembrane domains are sufficient for structural and functional coupling of Ti to CD3.

The structural and functional interactions of individual chains of polypeptides comprising oligomeric membrane receptors are poorly understood. The TCR is a multi-chain complex consisting of a disulfide-linked heterodimer (Ti) associated with at least five invariant chains ( $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta_2$ ), which together constitute the CD3 complex (1–3). On most T cells, the Ti subunit consists of an  $\alpha/\beta$  heterodimer, Ti  $\alpha/\beta$ , which is responsible for the recognition of antigen in the form of peptide fragments associated with MHC molecules on the surface of antigen presenting cells (4, 5). On a small subpopulation of T cells, an alternative form of the Ti subunit, Ti  $\gamma/\delta$ , is expressed in association with CD3 (6–8), although the antigen specificity of Ti  $\gamma/\delta$  has not been definitively established. The CD3 chains are thought to couple Ti to intracellular signal transduction mechanisms that are activated by TCR stimulation, including the phosphatidylinositol pathway and a tyrosine kinase pathway (9, 10). Structurally, the longer cytoplasmic (CYT)<sup>1</sup> domains of CD3 (40–113 amino acids) compared with those of the Ti chains (five amino acids) are more consistent with this signaling function for CD3 (1, 11). Moreover, stimulation of CD3 with mAbs mimics the effects of the binding of antigen or anti-Ti mAbs to Ti in inducing signal transduction events and T cell activation (9, 12, 13).

Ti and CD3 are intimately associated on the plasma membrane as demonstrated by a variety of methods. Ti and CD3

cointernalize in response to mAbs against either structure (14). Under appropriate conditions of solubilization, Ti and CD3 can be coimmunoprecipitated (8, 15, 16). They can also be chemically crosslinked on unstimulated T cell lines (17, 18).

Previous studies with somatic cell mutants have shown that surface expression of CD3 is dependent on its proper assembly with Ti  $\alpha$  and  $\beta$  (19–21) and on  $\zeta$  chain expression (22, 23). Our study was undertaken to define the domain(s) of the Ti chains that are responsible for the structural and functional association with CD3. The varying ability of different detergent concentrations to preserve the integrity of the TCR complex during solubilization has suggested that the hydrophobic transmembrane (TM) domains are important in Ti/CD3 association (8). Interestingly, the Ti and CD3 chains contain unusually positioned, basic or acidic residues within their otherwise hydrophobic TM domains. These amino acids are highly conserved across species (1, 2, 24). Ti  $\alpha/\beta$  as well as Ti  $\gamma/\delta$  TM domains contain the basic amino acids lysine and arginine, whereas the TM domains of CD3 contain residues of the opposite charge, glutamate and aspartate. It has been proposed that these charged residues may be responsible for Ti/CD3 interaction through the formation of salt bridges (25). We have demonstrated that Lys 290 in Ti  $\beta$  is a critical residue. Site-directed mutagenesis of Lys 290 to residues of conserved charge or conformation prevented surface expression of Ti/CD3 (26). This finding was confirmed by Alcover et al. (27), who in addition, showed that at least two Ti  $\beta$  mutants at Lys 290 (to Ala or Gln) could dimerize with the  $\alpha$  chain intracellularly, but association with CD3 was abrogated. Similarly, no surface expression of the TCR

<sup>1</sup> Abbreviations used in this paper: CYT, cytoplasmic; EC, extracellular; ER, endoplasmic reticulum; IP, inositol phosphate; SFV, Friend spleen focus-forming viral; TM, transmembrane.

was obtained when Arg 121 and Lys 126 in the TM region of Ti  $\alpha$  were altered by mutagenesis (28, 29). Interestingly, the recent report by Blumberg et al. (29) showed that only when both basic residues in the TM domain of Ti  $\alpha$  were mutated was  $\alpha\beta$  heterodimer formation and TCR/CD3 surface expression blocked. Mutation of either the Arg to Gly or Lys to Ile alone did not interfere with  $\beta$  or CD3 association nor with the response of the resulting TCR to antibody stimulation. Although mutations to other amino acids other than Gly and Ile have not been tested and could possibly have more deleterious effects, it appears that in the Ti  $\alpha$  chain, either one of the basic residues in the TM domain was sufficient to allow interaction with the CD3 chains. These studies demonstrate that these basic residues are required at their particular positions but provide little insight on their role in the interaction between Ti and CD3.

To define the structural requirements for Ti/CD3 chain association and signal transduction, chimeric Ti molecules were constructed using the extracellular (EC) domain of CD8 $\alpha$  linked to the regions of Ti  $\alpha$  and  $\beta$  containing the TM domains. CD8 is a transmembrane protein expressed specifically on the surface of T cells and, like Ti, exists as a dimer in addition to its monomer and multimer forms (30). Chimeric CD8/Ti  $\alpha$  and CD8/Ti  $\beta$  were transfected into a Jurkat mutant that is deficient in endogenous Ti $\beta$  transcription and as a consequence fails to express cell surface CD3. Our results demonstrate that Ti is physically and functionally linked to CD3 via the COOH termini containing their TM domains.

## Materials and Methods

**Cell Lines.** The human leukemic T cell line Jurkat (clone E6-1) and its Ti $\beta$ -negative derivative, J.RT3-T3.5, were maintained in complete medium, which consists of RPMI 1640 supplemented with 5% FCS, glutamine, penicillin G, and streptomycin (Irvine Scientific). Jurkat.CD8 (transfected with CD8 $\alpha$ ) was passaged in complete medium containing Geneticin (Gibco Laboratories, Grand Island, NY) at 1 mg/ml. J.RT3-T3.5 was sequentially transfected with the chimeric  $\alpha$  and  $\beta$  chain, and 10.23 is a transfectant that expresses both chimeras.

**Monoclonal Antibodies.** The following mouse mAbs were used: both OKT8 (American Type Culture Collection, Bethesda, MD) and Leu-2A (Becton Dickinson Monoclonal Center, Mountain View, CA) recognize external CD8 epitopes. Anti-Leu-4 (Becton Dickinson Monoclonal Center) is reactive with CD3. W6/32 recognizes an invariant determinant expressed on human HLA class I antigens (31). C305 is specific for Jurkat E6-1 Ti $\beta$  (19). MOPC 195, an IgG2a, and MOPC 104E, an IgM (both from Litton Biogenetics, Kensington, MD), were used as control antibodies in FACS<sup>®</sup> analyses (Becton Dickinson & Co.). Ascitic fluids of mAbs were used at a final dilution of 1/1,000 (a saturating concentration) in all experiments.

**Construction and Expression of Chimeric Ti.** The cDNAs and amino acid sequences for Ti  $\alpha$  and  $\beta$  (Jurkat) and CD8  $\alpha$  have been described (30, 32, 33). The plasmid pSV7d-CD8 $\alpha$  (generously provided by Dan Littman, University of California, San Francisco) contains the CD8  $\alpha$  chain cDNA in the EcoRI site. A HindIII site was introduced into the unique NaeI site present near the TM region of the CD8 cDNA by the insertion of an 8-bp HindIII linker (Boehringer Mannheim Biochemicals, Indianapolis, IN). Ti  $\alpha$

cDNA contains a HindIII site near the TM region. The COOH terminus of CD8 (in pSV7d) from HindIII to EcoRI was replaced by a 200-bp fragment of Ti  $\alpha$  containing the TM and CYT domains to generate CD8/Ti $\alpha$ . This chimeric cDNA was cloned as an EcoRI fragment into the vector SFFV.SVneo (a gift of Dennis Loh, Washington University, St. Louis, MO) immediately 3' of the Friend spleen focus-forming viral (SFFV) LTR. An XbaI-BamHI fragment containing Ti  $\beta$  was excised from the plasmid pTi $\beta$ Neo (20) and subcloned into pUC 19. This subclone was linearized by partial digestion with Eco109 and ligated with an adaptor containing a HindIII site. Introduction of the HindIII site results in valine 271 in the EC region being substituted by leucine. However, the translational reading frame is preserved in the final construct. Plasmid pCD8/Ti $\beta$  was created by replacing the Ti  $\beta$  sequences 5' of the new HindIII site with the same CD8 EC segment used in the chimeric  $\alpha$  construct. Subsequently, an EcoRI-BamHI fragment containing the SFFV long terminal repeat and CD8/Ti $\beta$  was transferred into the expression vector pSV2gpt (34).

The CD8/Ti $\alpha$  and CD8/Ti $\beta$  plasmids bearing selectable markers for resistance to neomycin or mycophenolic acid were transfected into cells by electroporation using a gene pulser (Bio-Rad Laboratories, Richmond, CA) at 300 V and a capacitance of 500  $\mu$ F, with 20  $\mu$ g of plasmid DNA per 10<sup>7</sup> cells. Neo gene transfectants were selected in medium containing Geneticin at 2 mg/ml. Gpt transfectants were isolated by selection using xanthine (50  $\mu$ g/ml), hypoxanthine (3  $\mu$ g/ml), and mycophenolic acid (1.2  $\mu$ g/ml). Xanthine and hypoxanthine were purchased from Sigma Chemical Co. (St. Louis, MO), and mycophenolic acid is from Calbiochem-Behring Co. (La Jolla, CA).

**Flow Cytometry.** Cells were analyzed for expression of CD3 and the CD8 epitope by cell surface immunofluorescence techniques as described (19). For double staining, cells were stained directly with FITC-Leu-2A and PE-Leu-4. FITC- and PE-conjugated control mouse IgGs were used for background staining. Conjugated mAbs were from Becton Dickinson & Co.

**Northern Blot Analysis.** Total RNA was isolated from cells using the guanidine thiocyanate method and centrifuged through a CsCl cushion (35). RNA (15  $\mu$ g per lane) was separated electrophoretically on a 1% formaldehyde agarose gel and transferred to MSI nylon (Fisher Scientific). The <sup>32</sup>P-labeled probe was a 600-bp cDNA fragment corresponding to the extracellular region of CD8.

**Surface Iodination and Immunoprecipitation.** Cells were labeled with <sup>125</sup>I using lactoperoxidase/glucose oxidase (Sigma Chemical Co.) as described (19). The lysis buffer consists of 150 mM NaCl, 10 mM Tris-HCl, pH 7.8, with 1 mM PMSF and aprotinin (18  $\mu$ g/ml) added as protease inhibitors together with either 1% digitonin plus 0.12% Triton-X or 1% NP-40. Preclearing and immunoprecipitation of cell lysates and analysis of proteins on SDS-PAGE were performed as described previously (36) with the following modifications. Rabbit anti-mouse IgG (Zymed Laboratories, So. San Francisco, CA) was bound to formalin-fixed *Staphylococcus aureus* (Calbiochem-Behring Co.) and then armed with normal mouse serum, OKT8, or anti-Leu-4.

**Inositol Phosphate Assay.** Inositol phosphates (IPs) were quantitated as described (37). Cells loaded with [<sup>3</sup>H]myo-inositol were incubated with mAb (1/1,000 dilution of ascites) or media as control, in the presence of 10 mM LiCl for 30 min at 37°C and then lysed. Samples were performed in triplicate. IPs were eluted from an anion exchange resin column, and the amount of labeled material was determined by scintillation counting.

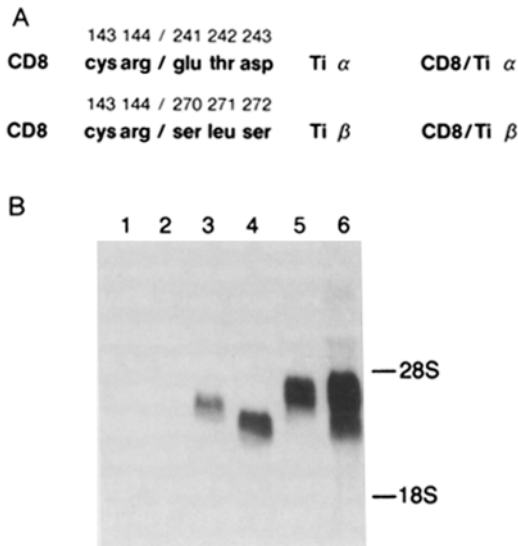
**[Ca<sup>2+</sup>]<sub>i</sub> Measurement by Fluorimetry.** Fluorimetry using indo-1 has been previously described (38).

**IL-2 Bioassay.** 24-well tissue culture dishes were coated with

rabbit anti-mouse Ig (Zymed Laboratories) at 100  $\mu\text{g}/\text{ml}$  in carbonate buffer, pH 9.5 (15 mM  $\text{Na}_2\text{CO}_3/35$  mM  $\text{NaHCO}_3/0.02\%$   $\text{NaN}_3$ ). The wells were blocked with medium plus 10% FCS followed by incubation with the appropriate mouse mAbs. Cells were cultured in complete medium at a density of  $3 \times 10^6$  cells/ml in the coated culture dishes in the presence of PMA (10 ng/ml) or PMA plus ionomycin (1  $\mu\text{M}$ ), for 24 h. Culture supernatants were collected and assessed for IL-2 by the ability to support the growth of the IL-2-dependent indicator cell line CTLL-2.20 using the MTT colorimetric assay as described (39).

## Results

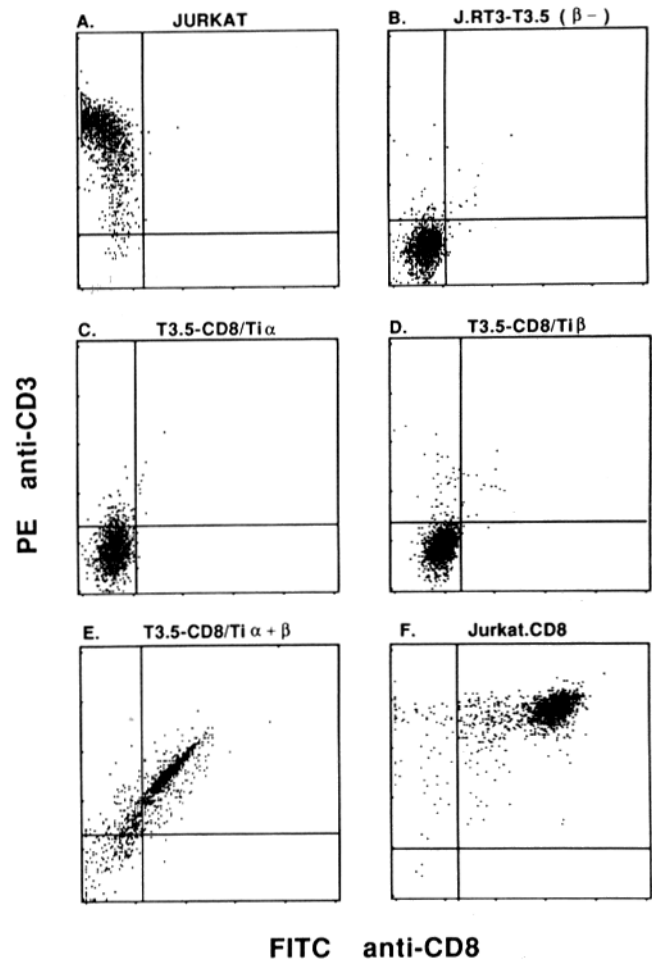
**Construction and Expression of the CD8/Ti Chimeras.** Hybrid Ti  $\alpha$  and  $\beta$  chains were constructed by joining the CD8  $\alpha$  chain EC domain to the COOH terminus of either Ti  $\alpha$  or  $\beta$ . Since the precise limits of the TM domains of the Ti chains at the protein level have not been defined, chimeric chains were constructed near the boundary of the Ti constant region exon 3, which encodes the TM domain of Ti  $\beta$  and both the TM and CYT domains of Ti  $\alpha$ , respectively (40, 41). Thus, these COOH-terminal segments contain the putative TM domain, the five amino acids of the CYT domain, as well as 14–16 residues of the Ti  $\alpha$  and  $\beta$  EC domains preceding the TM domain sequence. The amino acid sequence at the cloning junction is shown in Fig. 1 A.



**Figure 1.** Construction and expression of the CD8/Ti chimeric chains. (A) Chimeric Ti chains were constructed by linking the same cDNA fragment encoding the EC region of CD8  $\alpha$  to the COOH termini of Ti  $\alpha$  and  $\beta$  beginning at the junction of constant region exon 3, which encodes the TM domain in each case. The amino acid sequences at the CD8 and Ti junction are shown. The number above each residue refers to its position in the wild-type sequence. In the construction of CD8/Ti $\beta$ , a conservative change was made converting amino acid 271 in Ti $\beta$  from val to leu. (B) Northern blot analysis of RNA from the parental and transfected cell lines. Total RNA (15  $\mu\text{g}$  per lane) was probed with a  $^{32}\text{P}$ -labeled cDNA fragment corresponding to the CD8 EC domain. Lane 1, J.RT3-T3.5 (Ti $\beta$ - mutant of Jurkat); lane 2, Jurkat; lane 3, Jurkat.CD8; lanes 4–6, J.RT3-T3.5 transfected with chimeric CD8/Ti  $\alpha$ , CD8/Ti  $\beta$ , or CD8/Ti  $\alpha$  plus CD8/Ti  $\beta$ , respectively.

The CD8/Ti $\alpha$  and CD8/Ti $\beta$  plasmids were transfected into J.RT3-T3.5, a mutant of the human T cell leukemic line Jurkat, which fails to express the TCR complex due to deficient endogenous Ti  $\beta$  chain transcription (20). To determine if the chimeras could form homodimers, since CD8 does exist in homodimeric form, cells were transfected with single chimeric chains as well as with both chimeric chains in a sequential manner. In addition, wild-type Jurkat cells were transfected with the full-length CD8  $\alpha$  chain cDNA to produce the cell line Jurkat.CD8.

Expression of transcripts encoding the transfected chains was assessed by Northern blot analysis of total RNA isolated from representative transfectants. Jurkat and its  $\beta$  chain-deficient derivative, J.RT3-T3.5, are CD4 $^+$  lines and do not express endogenous CD8 mRNA (Fig. 1 B, lanes 1 and 2). Jurkat.CD8 expressed CD8  $\alpha$  mRNA (lane 3). Both single chimeric chain transfectants expressed high levels of the relevant transcripts (lanes 4 and 5). The double transfectant, clone



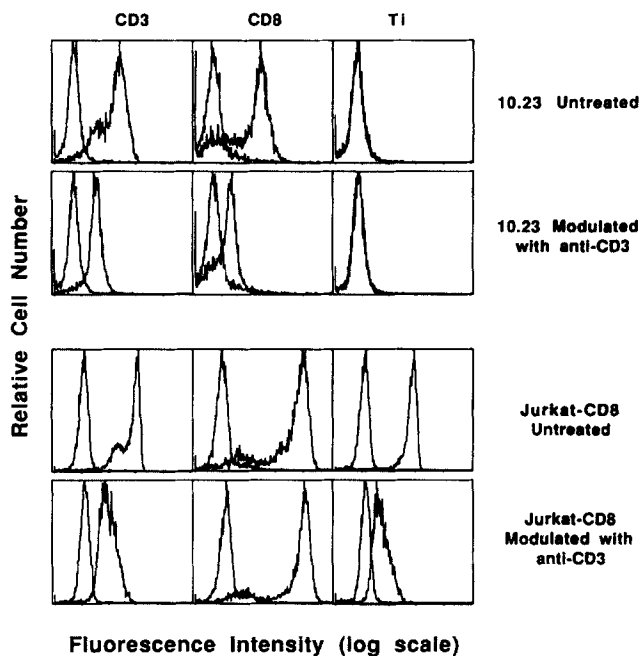
**Figure 2.** Double immunofluorescence staining for cell surface CD8 and CD3. Cells were stained with FITC-conjugated anti-CD8 mAb (Leu-2A) and PE-conjugated anti-CD3 mAb (anti-Leu-4) simultaneously and analyzed by flow cytometry. As a negative control, cells were stained with FITC- and PE-conjugated mouse control IgG. The panels are divided into quadrants to define negative, single-positive, or double-positive cells.

10.23 (lane 6), expressed high levels of both chimeric transcripts.

These six clonal cell lines in Fig. 1 B were analyzed for surface expression of CD3 and the EC domain of CD8 by double immunofluorescence staining with FITC-conjugated anti-CD8 and PE-conjugated anti-CD3 mAbs (Fig. 2). Jurkat expressed high levels of CD3 only (Fig. 2 A). In contrast, the Ti $\beta$ -negative mutant did not express surface CD3 or CD8 (Fig. 2 B). The single chimeric chain transfectants that did synthesize abundant chimeric mRNAs (Fig. 1 B) failed to express surface CD8 (Fig. 1, C and D). Moreover, cell surface expression of CD3 was not reconstituted in these cells. The failure to detect CD8 and CD3 on screening multiple single chimeric chain transfectants that were positive at the RNA level suggest that the individual chimeric chain behaved more like individual wild-type Ti chains than CD8 in their requirements for surface expression and did not form homodimers. However, when both chimeras are expressed in a single cell, as in clone 10.23, not only were CD8 antigenic determinants expressed, but surface CD3 was restored as well (Fig. 2 E). Expression of the CD8 and CD3 determinants on 10.23 was stoichiometric, suggesting that they are expressed on the same molecular complex. In comparison, Jurkat.CD8 did not demonstrate this linear relationship (Fig. 2 F) since wild-type CD8 is expressed independent of CD3 in this transfectant as it is in other cells that normally express CD8. These results suggest that the chimeric CD8/Ti receptor is expressed as a heterodimer that is physically associated with CD3.

**Physical Association of CD8/Ti and CD3.** To determine if the chimeric CD8/Ti receptor is physically linked to the CD3 complex, two additional lines of investigation were used. The TCR complex is internalized in response to soluble anti-Ti or anti-CD3 mAbs (14). Therefore, it was of interest to determine whether the CD8/Ti chimeric receptor would coinitalize with CD3. Cells were incubated for 16 h at 37°C in the presence or absence of an anti-CD3 mAb (anti-Leu-4), and then stained by indirect immunofluorescence for CD3, CD8, and Ti expression. mAb C305 specifically detects Jurkat Ti  $\beta$ . Untreated clone 10.23 did not stain with C305, verifying that this clone has a  $\beta$  chain-negative background (Fig. 3). Pretreatment with anti-Leu-4 resulted in diminished expression of both CD3 and the CD8 determinant on clone 10.23. With Jurkat.CD8, Ti and CD3 coinitalized after anti-CD3 treatment but the expression of CD8 was not affected.

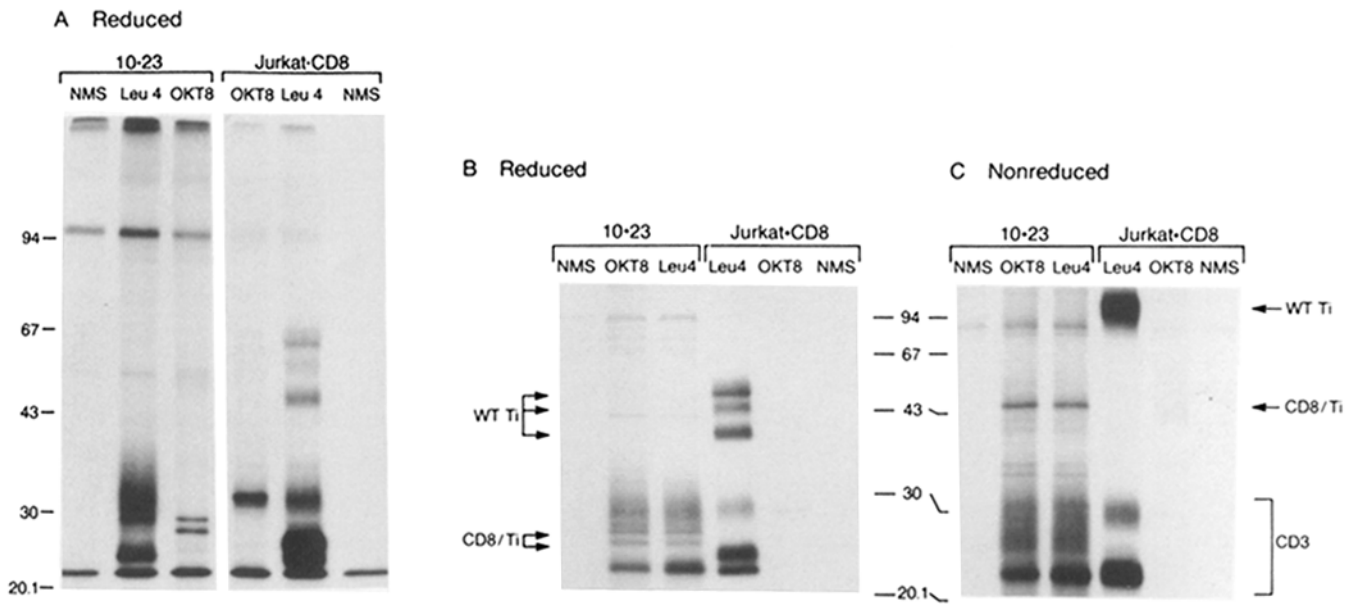
Further evidence that the chimeras are physically coupled to CD3 was the ability of the two complexes to coimmunoprecipitate with either anti-CD8 or anti-CD3 mAb. Surface radioiodinated cells were solubilized in NP-40, in which CD8 is more soluble (Fig. 4 A), or in digitonin buffer (Fig. 4, B and C), which preserves the Ti/CD3 association. From NP-40 lysates of 10.23, anti-CD8 immunoprecipitated the chimeric chains of 27 and 28 kD, consistent with the predicted molecular masses of CD8/Ti  $\alpha$  and  $\beta$  (Fig. 4 A). In NP-40, these bands were immunoprecipitated independent of CD3. Under nonreducing conditions, the chimeric chains were immunoprecipitated as disulfide-linked dimers (data not shown).



**Figure 3.** Comodulation of CD8/Ti and CD3 with anti-CD3 antibody. Cells were incubated for 16 h at 37°C with anti-CD3 (anti-Leu-4) ascites (1:1,000 dilution) or with no antibody (control). Cells were stained for CD3, CD8, or Ti with the appropriate mAb (anti-Leu-4, OKT8, and C305, respectively) using FITC-conjugated goat anti-mouse Ig as second antibody and then analyzed on a FACScan<sup>®</sup> flow cytometer (Becton Dickinson & Co.). The left histogram in each panel represents a negative control cells stained with a nonreactive mouse Ig.

From Jurkat.CD8, anti-CD8 immunoprecipitated only a 32-kD band corresponding to the CD8 monomer (Fig. 4 A), which has a mobility similar to that of the CD3  $\gamma$  chain. Although Ti chains did coprecipitate with CD3 from Jurkat.CD8 in this experiment using NP-40, more reliable coprecipitation is generally obtained using lysates prepared in digitonin. In digitonin buffer, both anti-CD8 and anti-CD3 immunoprecipitated identical proteins consisting of the chimeric chains as well as the CD3 complex of molecular mass range of 20 to 30 kD from 10.23 lysates (Fig. 4, B and C). The CD3 pattern in 10.23 differs from that in Jurkat.CD8. On further analysis, it was found that the  $\delta$  chain in 10.23 has reduced mobility and migrates as a broad band partly buried within the 28–30-kD CD3  $\gamma$  chain. This difference in mobility is most likely due to a difference in glycosylation, based on studies with a CD3  $\delta$  chain-specific antibody (data not shown). The chimeric disulfide-linked dimer seen under nonreducing conditions (Fig. 4 C) is consistently observed as a band of  $\sim$ 45 kD in repeated experiments. Thus, the chimeric  $\alpha$  and  $\beta$  chains are disulfide linked, but migrate anomalously under nonreducing conditions.

In contrast to the studies with the 10.23 cell line, anti-CD3 and anti-CD8 immunoprecipitates from the Jurkat.CD8 clone were strikingly different. Anti-CD3 mAb precipitated the wild-type Ti chains (the 40–49-kD bands) as well as the  $\gamma$  (28–30 kD),  $\delta$  (23 kD), and  $\epsilon$  (22 kD) chains of CD3, but not CD8 (Fig. 4 B and C). This CD3 immunoprecipi-

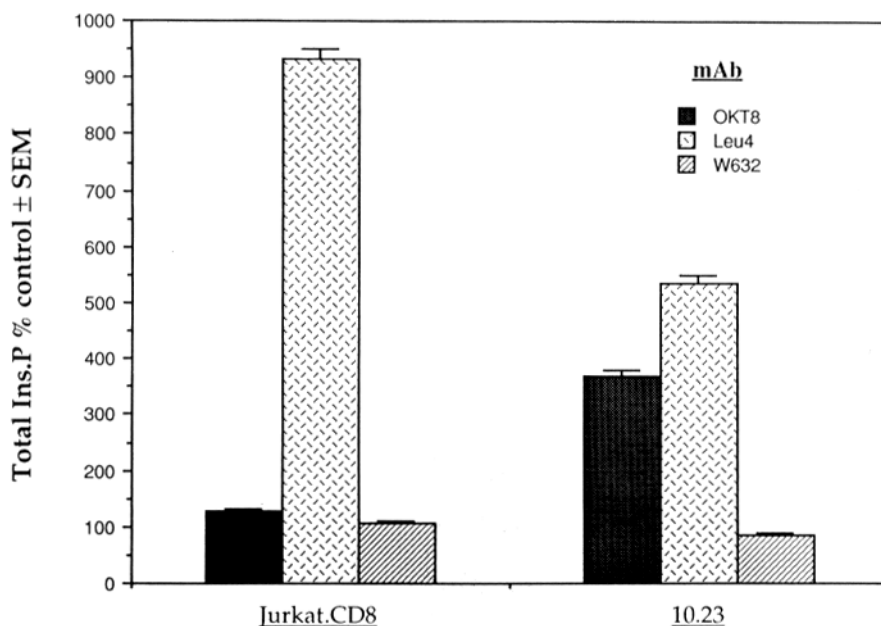


**Figure 4.** Coimmunoprecipitation of surface CD8/Ti and CD3. Cell surface proteins were labeled with  $^{125}\text{I}$  and the cells lysed in 1% NP-40 buffer (A), or in 1% digitonin lysis buffer (B and C), which preserves the Ti/CD3 association. Cell lysates were immunoprecipitated with normal mouse serum (NMS), anti-CD8 (OKT8), or anti-CD3 (Leu-4) mAb. Immunoprecipitates were analyzed on 10% SDS-PAGE under reduced (A and B) or nonreduced (C) conditions. Wild-type (WT) Ti, CD8/Ti, CD3, and the positions of the relative molecular mass standards (in kD) are indicated.

tate is identical to those that have previously been shown from this laboratory using wild-type Jurkat cells that do not express CD8 (8, 38). In the CD3 immunoprecipitate of the Jurkat.CD8 lysate (Fig. 4 B), the two highest bands migrating at 45 and 49 kD correspond to different glycosylated forms of Ti  $\alpha$ , as was shown in previous studies, and the lowest of the three bands is Ti  $\beta$ . None of the Ti or the CD3 chains were visualized in immunoprecipitates preserved with the anti-CD8 mAb (Fig. 4 B and C) in five separate experiments. Note that the 28-kD faint band seen under reducing conditions

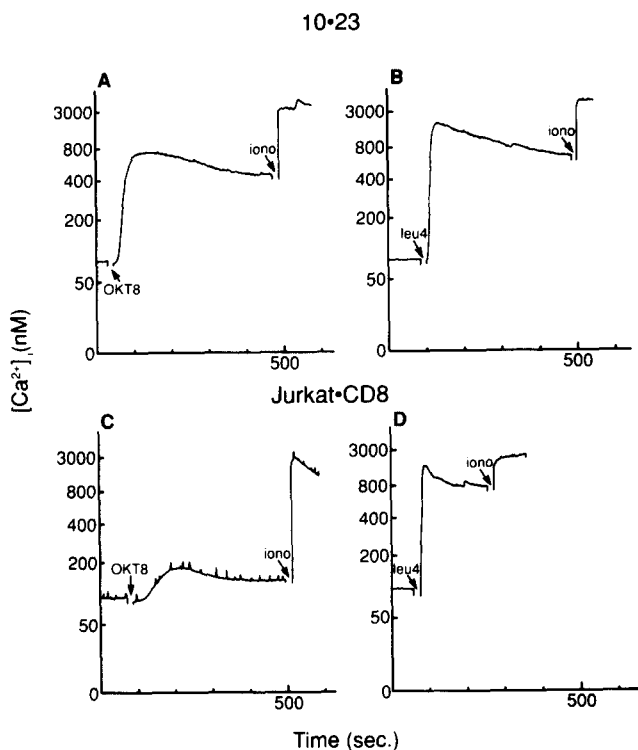
in the anti-CD8 immunoprecipitate migrates as a faint diffuse band with higher apparent molecular mass under nonreducing conditions and therefore represents CD8 (compare OKT8 lanes in panels B and C). CD8 apparently has low solubility in digitonin. Collectively, these results demonstrate that the CD8/Ti chimera is physically associated with CD3 on 10.23 but that normal CD8 does not similarly interact with CD3 on Jurkat.CD8.

*Functional Coupling of CD8/Ti to CD3.* Having demonstrated that the COOH termini of Ti  $\alpha$  and Ti  $\beta$  containing



**Figure 5.** Inositol phosphate generation upon stimulation of the chimeric receptor. Cells loaded with  $^3\text{H}$ myo-inositol were incubated with or without (control) mAb for 30 min at  $37^\circ\text{C}$  in the presence of  $\text{LiCl}$ , and then lysed. Water-soluble  $^3\text{H}$ inositol phosphates were extracted and counted.

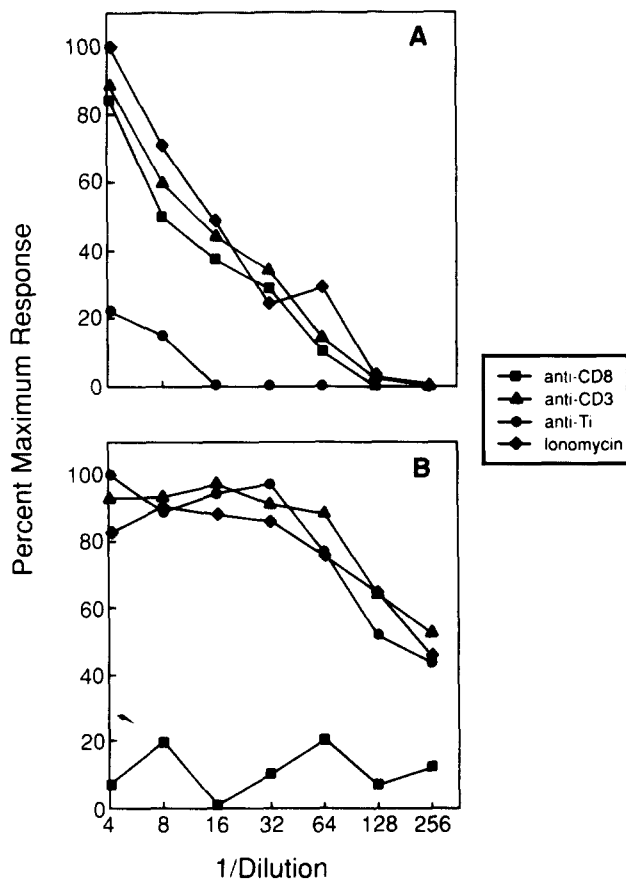
the TM domains sufficient for the chimeric receptor association with CD3, we next determined if this structural coupling to CD3 also resulted in a functional interaction between the chimeric receptor and CD3. mAbs reactive with Ti or CD3 but not CD8 can mimic the effects of antigen and serve as agonists to stimulate signal transduction events as well as cellular activation responses (9). The ability of the CD8/Ti receptor to transduce signals via the phosphatidylinositol pathway upon stimulation with agonistic mAbs was assessed. The generation of inositol phosphates upon stimulation with anti-CD8 and anti-CD3 was assessed in 10.23 and Jurkat.CD8 (Fig. 5). 10.23 could be stimulated by both anti-CD8 and anti-CD3 to increase inositol phosphate levels to  $370 \pm 11\%$  and  $537 \pm 11\%$  of unstimulated cells, respectively. This response was specific since W6/32, an antibody to MHC class 1, failed to induce increases in inositol phosphates. In contrast, Jurkat.CD8 responded normally to anti-CD3 but failed to respond well to anti-CD8, even though it expressed four- to fivefold more CD8 than the chimeric CD8/Ti receptor on 10.23. The smaller increase of inositol phosphates observed in 10.23 in response to anti-CD3 can be attributed to its lower level of CD3 expression (threefold lower than Jurkat.CD8; see Fig. 3) since the magnitude of inositol phosphate generation is directly related to the level of TCRs engaged on other cells (Graber, Bockenstedt, and Weiss, unpublished observations).



**Figure 6.** Induction of  $[Ca^{2+}]_i$  increase. Cells loaded with indo-1 were stimulated with OKT8 or anti-Leu-4 ascites (1:1,000 dilution) and analyzed for  $[Ca^{2+}]_i$  changes by fluorimetry. To ensure proper loading of the cells with the indo-1 fluorescent dye and releasability of  $Ca^{2+}$  stores, cells were treated with  $1 \mu M$  ionomycin. Arrows indicate the addition of mAb or ionomycin.

As a consequence of the generation of inositol phosphates in T cells, increases in cytoplasmic free calcium ( $[Ca^{2+}]_i$ ) are observed (37). To measure calcium responses, 10.23 and Jurkat.CD8 were loaded with indo-1, a calcium-sensitive fluorescent dye, and stimulated with mAbs. Changes in  $[Ca^{2+}]_i$  were monitored by fluorimetry. In 10.23, anti-CD8 induced a rapid and large rise in  $[Ca^{2+}]_i$ , with a peak response of 609 nM over baseline (Fig. 6 A). Only a very small increase in  $[Ca^{2+}]_i$  was observed with Jurkat.CD8 in response to anti-CD8 (C) although the cells were capable of responding normally to anti-CD3 (D). The small increase in  $[Ca^{2+}]_i$  in Jurkat.CD8 was only observed at the highest concentration of anti-CD8 used.

As a final assay for chimeric CD8/Ti receptor function, these cells were tested for IL-2 production in response to stimulating mAbs. Jurkat.CD8 and 10.23 cells were stimulated for 18–24 h with mAb plus PMA. PMA is required for TCR-induced IL-2 production in Jurkat cells (42). The culture supernatants were harvested and assessed for IL-2 con-



**Figure 7.** Induction of IL-2 production. (A) 10.23 and (B) Jurkat.CD8 cells were stimulated with the mAbs C305 (anti-Ti) (●), anti-Leu-4 (anti-CD3) (▲), or OKT8 (anti-CD8) (■), or with ionomycin ( $1 \mu M$ ) (◆), all in the presence of PMA ( $10 \text{ ng/ml}$ ). IL-2 secretion was determined by the ability of culture supernatants of stimulated cells to support the growth of CTL20 indicator cells as assessed in the MTT colorimetric assay. Survival of the indicator cell is proportional to the absorbance at 570 nm in this assay. Results were normalized to the maximal response in this assay.

tent by their ability to support the growth of CTLL-20, an IL-2-dependent cell line. Clone 10.23 produced IL-2 in response to either anti-CD8 or anti-CD3 (Fig. 7 A). mAb C305 served as a negative control since 10.23 lacks Ti  $\beta$ . In contrast, anti-CD8 did not induce IL-2 production by Jurkat.CD8, although these cells were capable of responding to either C305 or anti-Leu-4 (Fig. 7 B). In contrast to previous studies with Jurkat cells or with Jurkat.CD8, immobilization of mAb was required to induce IL-2 production by 10.23. We presume that mAb in solution did not stimulate IL-2 production in 10.23 because of the lower number of TCRs that are expressed on 10.23 and that a proportion of these receptors are internalized in response to stimulation with soluble mAbs. 10.23 expresses threefold fewer receptors than Jurkat.CD8 as determined by flow cytometry analysis (also see Fig. 3). Note that the potential of these two cells to produce IL-2, as measured by their maximal responses to the calcium ionophore ionomycin plus PMA, also differs. Although 10.23 produces substantially less IL-2 than Jurkat.CD8, 10.23 produced nearly maximal levels of IL-2 when the CD8/Ti chimeric receptor was stimulated. Collectively, our results demonstrate that the CD8/Ti chimeric receptor is functionally coupled to the CD3 complex.

## Discussion

As a first step towards determining the region essential for Ti and CD3 association and function, we have constructed a chimeric TCR with the COOH-terminal regions of Ti  $\alpha$  and  $\beta$  linked to the EC domain of CD8, a T cell-specific surface glycoprotein. Here, we demonstrate that expression of both chimeric chains restores CD3 plasma membrane expression on a mutant of the T cell leukemic line Jurkat that lacks functional endogenous Ti $\beta$  transcripts. In addition to defining domains of the Ti  $\alpha$  and  $\beta$  chains involved in structurally linking Ti to CD3, these domains were shown to functionally link Ti to signal transduction mechanisms associated with CD3. These studies support the role of CD3 in mediating TCR transmembrane signaling.

Although CD8 can form homodimers on T cells and on fibroblasts (30), transfection of CD8/Ti $\alpha$  or CD8/Ti $\beta$  alone did not reconstitute surface expression of CD3, nor was the CD8 epitope detected by mAb OKT8 by FACS<sup>®</sup> analysis. Thus, the chimeras behaved more like the individual wild-type Ti chains than CD8 since they did not form homodimers. This suggests that the COOH-terminal domains of CD8 and Ti are not interchangeable. In addition, heterodimers of CD8/Ti  $\beta$  and endogenous Ti  $\alpha$  were not expressed, indicating that the EC domains do influence Ti  $\alpha$  and Ti  $\beta$  association. The EC region of Ti  $\alpha$  is composed of two Ig-like domains and is much larger (232 amino acids) than the EC region of CD8 (150 amino acids), which contains only a single Ig-like domain. The different EC domains may not be compatible, possibly due to steric hindrance.

We found that both chimeric chains were required to express the chimeric Ti and to restore CD3 onto the cell surface. Moreover, this association between the chimeras and CD3 showed a stoichiometric relationship. That the two com-

plexes were physically linked was confirmed by their ligand-induced cointernalization and their coimmunoprecipitation. The chimeric chains formed interchain sulfhydryl bonds, presumably through cys 143 from the EC domain of CD8, the only cysteine that is not in the Ig-like fold. The cysteines in Ti  $\alpha$  and  $\beta$  that are thought to be responsible for interchain disulfide linkages were eliminated in the construction of the chimeras.

Our data demonstrate that the physical association between CD3 and the CD8/Ti chimeras resulted in a functional interaction as well. The chimeric CD8/Ti could effectively transmit signals for T cell activation through the phosphatidylinositol pathway that is coupled to CD3. Anti-CD8 mAb was able to induce an increase in inositol phosphates and a large rise in  $[Ca^{2+}]_i$ , as well as IL-2 production through the chimeric receptor but not through wild-type CD8. Thus, by linking the EC domain of CD8 to the COOH termini of the Ti  $\alpha$  and  $\beta$  chains, a functional interaction with CD3 was established. These data are consistent with the notion that the role of CD3 is to couple ligand-occupied Ti to intracellular signaling mechanisms.

These studies have defined a region within the COOH termini of Ti  $\alpha$  and  $\beta$  that contains all of the information required for structurally and functionally coupling the chimeric CD8/Ti to the CD3 complex. It is possible that the TM domains alone contained within the COOH-termini segments of Ti can mediate this association between CD8/Ti and CD3. It is less likely that the 14 and 16 amino acids extracellular to the putative published TM domains or the five amino acids of the CYT domains of both Ti chains contained in these chimeric constructs contribute to the association with the CD3 complex and signal transduction. Ti  $\gamma/\delta$  is also structurally and functionally associated with CD3 although its antigenic specificity is poorly understood. A comparison of the COOH termini of Ti  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  chains reveals little amino acid identity among the four chains in these EC and CYT regions, particularly in the comparison of  $\beta$  to  $\gamma$  and  $\alpha$  to  $\delta$ . In contrast, the predicted TM domains of Ti  $\alpha$  and Ti  $\delta$  are highly conserved (43, 44). Conservation of the sequences of the TM domains is also noted in the Ti  $\beta$  chain in chicken, whereas little homology is found in these EC and CYT regions (Craig Thompson, personal communication). One difficulty of studying subunit interactions of an oligomeric complex such as the TCR can be circumvented by studying the interaction in fibroblast cells that do not synthesize any of the TCR components. Although the physical interactions identified cannot be accompanied by functional studies, this system allows the dissection of a complex receptor into smaller units whose interactions could provide clues on how the whole complex is assembled. By transfecting pairs of TCR chains into COS cells, Manolios et al. (45) demonstrated that Ti  $\alpha$  associated with CD3  $\delta$ , and not  $\gamma$  or  $\zeta$ . By constructing a series of chimeric proteins using the Tac antigen fused to variable amounts of Ti  $\alpha$  TM and CYT domains, they were able to narrow down to a stretch of eight residues, including the two basic residues within the putative TM domain of  $\alpha$ , that still permitted assembly with  $\delta$ . Consistent with earlier reports, elimination of the two basic



residues in the TM region of  $\alpha$  abolished association with  $\delta$ . Replacement of the five CYT residues of  $\alpha$  with sequences from Tac had no effect on the association with  $\delta$ , but replacement of the TM region eliminated that association. Their findings are consistent with our data that point to the TM domain as being essential for physically and functionally coupling Ti to CD3. Our study is an initial step towards defining a minimal sequence involved in the Ti/CD3 interaction. Construction of additional chimeras currently underway and site-directed mutagenesis within the COOH-terminal regions of both Ti and CD3 would provide further insight into their interaction and perhaps elucidate the mechanism of signal transfer from ligand-bound Ti to CD3 and the associated intracellular signal transduction pathways.

There are two models to explain the functional coupling of CD8/Ti chimeric chains to signal transduction mechanisms in our studies. Upon ligand binding, Ti undergoes a conformational change that is transmitted via the COOH terminus to CD3. CD3 is then induced, presumably through allosteric changes, to activate the intracellular signaling mechanisms. Alternatively, the bivalent mAbs used as stimuli may be crosslinking CD3 complexes by interacting with the associated CD8/Ti chimera, and it is this effect of aggregation that initiates the cascade of signaling events. In theory, the use of monovalent Fab fragments could address this question. However, the inherent problems with Fabs, such as diminished avidity and aggregation at high concentrations that are necessary to mimic the effects produced by bivalent antibodies, precludes their usage in attempting to distinguish between the two models.

Another approach to studying chain interaction in a multi-subunit complex is through the use of synthetic peptides corresponding to particular domains of a subunit. Synthetic peptides corresponding to the TM domains of glycoprotein A were shown to complex specifically with native glycoprotein A chains in an in vitro bilayer system (46). This interaction

was not inhibited by synthetic peptides of the TM domains of glycoprotein C or the IL-2R Tac protein, which were similar in amino acid composition to that of glycoprotein A. Thus, there is precedent for the TM domains of at least one other plasma membrane oligomer, albeit a dimer, in mediating interchain associations.

In the assembly of the complete TCR complex, the  $\zeta$  chain is the limiting component, at least in the murine T cell line, 2B4. The other five chains ( $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\gamma$ , and  $\epsilon$ ) are synthesized in vast excess (47). These excess chains form pentamers within the endoplasmic reticulum (ER). A fraction of the pentamers then assemble into heptamers with the available  $\zeta$ . Both pentamers and heptamers are efficiently transported out of the ER and into the Golgi apparatus, but from there only heptameric complexes reach the cell surface (22). All other complexes are rapidly destroyed. It was shown that in T cells,  $\alpha$  chains associated with  $\beta$  and the CD3  $\gamma\delta\epsilon$  chains were destroyed in lysosomes (48). In fibroblasts, however, degradation of free  $\alpha$  chains occurred by an ER pathway. The region that targets unassembled  $\alpha$  chains for retention and rapid degradation was recently localized to the TM domain of the  $\alpha$  chain (49). These experiments and those reported here demonstrate that TM domains can contain not only the structural information for the insertion and anchoring of a protein in the membrane, but also sites for interaction with other subunits of a complex and, apparently, signals for degradation.

These studies have provided the first evidence to suggest that the regions surrounding and including the TM domains of an oligomeric plasma membrane receptor, the TCR, are involved not only in structural but also functional interactions between subunits of the receptor. The experimental approach used here should be applicable to other oligomeric receptor systems. Now that a region responsible for has been defined in the interaction between Ti and CD3, it should be possible to more precisely understand their structural and functional interactions.

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We thank Drs. Dan Littman, Anne Norment, Jim Fraser, and Gary Koretzky for their advice during these studies. We are grateful to Dr. Lewis Lanier for his generous gift of anti-Leu-4 mAb.

This work was supported in part by National Institutes of Health grant GM-39553. J. M. Turner was supported by a postdoctoral fellowship from the Science and Engineering Research Council, U.K.

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Received for publication 17 October 1990 and in revised form 4 December 1990.

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