Associations between Subunit Ectodomains Promote T Cell Antigen Receptor Assembly and Protect against Degradation in the ER

Thomas Wileman, Lawrence P. Kane, Juliana Young, Gerald R. Carson,* and Cox Terhorst

Division of Immunology, Beth Israel Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215; and *Protein Expression Group, T-cell Sciences Inc., Cambridge, Massachusetts 02139

Abstract. The T cell antigen receptor (TCR) is an oligomeric protein complex made from at least six different integral membrane proteins ($\alpha\beta\gamma\delta\epsilon$ and ζ). The TCR is assembled in the ER of T cells, and correct assembly is required for transport to the cell surface. Single subunits and partial receptor complexes are retained in the ER where TCR α, β, and CD3 δ chains are degraded selectively. The information required for the ER degradation of the TCR β chain is confined to the membrane anchor of the protein (Wileman et al., 1990*c*; Bonifacino et al., 1990*b*). In this study we show that the rapid degradation of the TCR β chain is inhibited when it assembles with single CD3 γ, δ, or ϵ subunits in the ER, and have started to define the role played by transmembrane anchors, and receptor ectodomains, in the masking proteolytic targeting information. Acidic residues within the membrane spanning domains of CD3 subunits were essential for binding to the TCR β chain. TCR β chains and CD3 subunits therefore interact via transmembrane domains. However, when sites of binding were restricted to the membrane anchor of the TCR β chain, stabilization by CD3 subunits was markedly reduced. Interactions between membrane spanning domains were not, therefore, sufficient for the protection of the β chain from ER proteolysis. The presence of the Cβ domain, containing the first 150 amino acids of the TCR ectodomain, greatly increased the stability of complexes formed in the ER. For assembly with CD3 ϵ, stability was further enhanced by the Vβ amino acids. The results showed that the efficient neutralization of transmembrane proteolytic targeting information required associations between membrane spanning domains and the presence of receptor ectodomains. Interactions between receptor ectodomains may slow the dissociation of CD3 subunits from the β chain and prolong the masking of transmembrane targeting information. In addition, the close proximity of TCR and CD3 ectodomains within the ER may provide steric protection from the action of proteases within the ER lumen.

THE T-cell antigen receptor (TCR)[1] plays a key role in the recognition of antigens by T-lymphocytes (T cells). The TCR functions at the surface of T cells as an oligomeric complex containing at least six different integral membrane proteins ($\alpha\beta\gamma\delta\epsilon$ and ζ). The α and β chains are structurally similar to immunoglobulins and have clone specific variable regions that bind peptide antigens presented to T cells. The invariant γ, δ, and ϵ subunits of the CD3 complex, and the disulfide-linked ζ dimer, control the intracellular transport of the TCR α and β chains, and allow them to communicate with signal transduction pathways in the cytosol (reviewed in Clevers et al., 1988; Klausner et al., 1990; Exley et al., 1991). In common with most multiple subunit membrane proteins, the assembly of the TCR takes place in the ER. Assembly is highly ordered and is characterized by the sequential addition of TCR α and β chains, and a ζ dimer, to a core complex of CD3 γ, δ, and ϵ subunits (Minami et al., 1987; Alarcon et al., 1988; Exley et al., 1991). Efficient transport from the ER only occurs for receptors that achieve a minimum 8-member ($\alpha\beta\gamma\delta\epsilon\epsilon\zeta$-$\zeta$) stoichiometry. Single subunits and partial receptor complexes are retained in the ER as assembly intermediates (Weiss and Stobo, 1984; Ohashi et al., 1985; Saito, 1987; Bonifacino et al., 1988; Sussman et al., 1988; Weissman et al., 1989; Wileman et al., 1990*a*; Carson et al., 1991; Hall et al., 1991).

In T cells, the individual components of the TCR are made in excess over the number that reach the cell surface (Minami, 1987). TCR subunits are retained in the ER and degraded by ER proteases (Wileman et al., 1990*b*; Lippincott-Schwartz et al., 1988; Chen et al., 1988). Interestingly, the proteolysis of TCR chains in the ER is carefully

Gerald Carson's present address is Repligen, Cambridge, MA 02139.

1. *Abbreviations used in this paper*: ADH, alcohol dehydrogenase; AFT, apoferritin; CA, carbonic anhydrase; endo H, endoglycosidase H, IL2R, interleukin 2 receptor; TCR, T cell antigen receptor; VSV, vesicular stomatitis virus.

regulated, and degradation rates vary with subunit structure and extent of receptor assembly. The CD3 γ and ϵ subunits are relatively resistant to proteolysis and survive for several hours. In contrast, the TCR α and β chains, and the CD3 δ subunit are highly susceptible to proteolysis in the ER, and are lost from cells within one hour of synthesis (Wileman et al., 1990*a*; Bonifacino et al., 1989). The short half-life of α, β, and δ is surprising when one considers that it takes up to 6 h for newly synthesized TCRs to leave ER and enter the Golgi apparatus (Alarcon et al., 1988). This high rate of degradation raises the question of how labile TCR subunits survive within the ER for incorporation into receptors that eventually reach the cell surface. This problem is, in part, solved during the assembe of the receptor. Two studies have shown that labile TCR chains are stabilized if they assemble with CD3 γ or ϵ subunits. The half-life of TCR β and CD3 δ chains rises, for example, some eightfold when they are complexed to CD3 γ or ϵ subunits (Wileman et al., 1990*a*; Bonifacino, 1989).

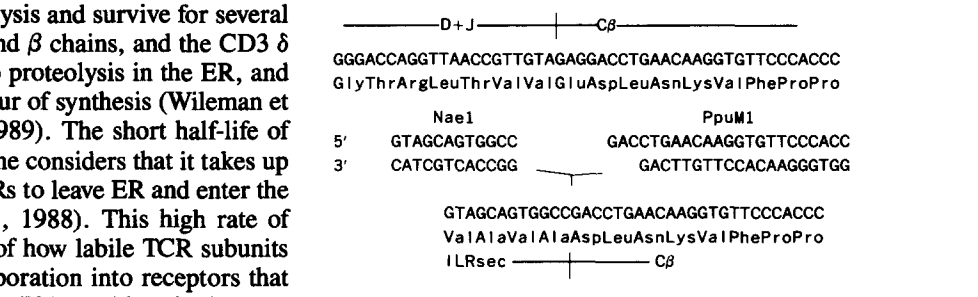
It is not known precisely how the binding of CD3 γ and ϵ subunits to labile TCR chains protects them from degradation in the ER. In the simplest model, the CD3 subunits may mask structural features that predispose unassembled proteins to the action of ER proteases. In the case of the TCR β chain, the structural information that leads to rapid ER degradation is contained within the membrane-spanning amino acids of the protein (Wileman et al., 1990*c*; Bonifacino et al., 1990*a*,*b*, 1991). In this present study we investigate the structural basis for the masking of this proteolytic targeting information during assembly with CD3 subunits. Unusually for integral membrane proteins, the membrane spanning domains of CD3 subunits contain acidic amino acids. We find that these acidic amino acids are essential for the binding of CD3 subunits to the TCR β chain and conclude that TCR and CD3 subunits interact via transmembrane domains. These interactions alone, however, were unable to stabilize the β chain within the ER. Significantly, the stability of complexes was markedly increased when the first 150 amino acids of the ectodomain (Cβ domain) of the TCR β chain was included for association with CD3 subunits. The results demonstrate that interactions between subunit ectodomains promote TCR assembly and stability within the lumen of the ER.

Materials and Methods

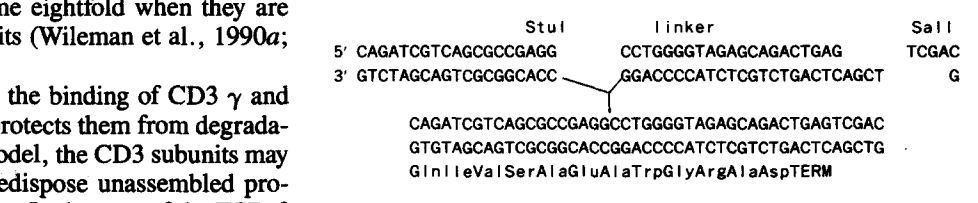
Construction of Fusion Proteins

The structures of the fusion proteins are shown as diagrams in Fig. 3. The construction of the β_{tm}ILRsec chimera has been described previously (Wileman et al., 1990*c*).

β_{tm}Cβ:ILRsec. The plasmid pYT35 (Yanagi et al., 1984) provided the c-DNA encoding the reading frame of the TCR β chain cloned from the human T cell line Jurkat. The plasmid pIL2R3 (Leonard et al., 1984) provided a c-DNA encoding the 55-kD α chain of the human IL2 receptor. The cDNA encoding the TCR β chain was subcloned into pUC19. A partial digest with PpuMI (base pair No. 444) opened the c-DNA at the base pairs encoding the diversity and joining amino acids (D+J junction) that lie between the constant (Cβ) and variable domains (Vβ). The resulting three base pair overhang was end-filled without loss of reading frame. The 5′-untranslated region of the cDNA, and the base pairs encoding the Vβ amino acids, were then removed using EcoRI. In separate experiments the nucleotides encoding the leader peptide and entire extracellular domain of the IL2 receptor were removed from pIL2R3 using a NaeI/EcoRI digest. The Nae I digest left an in-frame blunt end allowing the fragment to be subcloned into the opened TCR β c-DNA.



Cβ:ILRsec. The β_{tm}Cβ:ILRsec construct was subcloned into the EcoRI and HindIII sites of Bluescript. A digestion with SalI and StuI (base pair No. 818) removed the base pairs encoding the transmembrane anchor and cytoplasmic tail of the TCR β chain. A synthetic linker introduced a stop codon and closed the plasmid.



Site-specific Mutation

Charged amino acids within the membrane-spanning domains of the CD3 subunits were changed to alanine using oligonucleotide mediated mutation (Kunkel, 1985). A single base pair substitution (GAA to GCA) converted the glutamic acid (Glu 100) residue within the membrane anchor of the CD3 γ chain to alanine. A similar substitution (GAT to GCT) converted the aspartic acid residue (Asp 90) within the transmembrane anchor of CD3 δ to alanine, and the aspartic acid (Asp 115) residue within the membrane spanning domain of ϵ (GAC to GCC) to alanine.

Transfection

The TCR β chain and the CD3 proteins were expressed in CHO cells as described previously (Wileman et al., 1990*a*). For expression in COS cells, the reading frames encoding the CD3 proteins or the TCR/IL2R fusion proteins were subcloned into the pMNC8 expression vector described by Seed and Aruffo (1988). COS cells were transfected transiently using DEAE as described previously (Wileman et al., 1990*b*,*c*). For pulse-chase immunoprecipitation analysis, COS cells were trypsinized 16 h after transfection, mixed, and replated. This procedure generated a uniform population of cells and minimized dish to dish variation during the experiments.

Antibodies

Identity β-F1(TM) and AM 92.3 were provided by T-Cell Sciences (Cambridge, MA) and are specific for TCR β and IL2R, respectively. 7G7 was obtained from American Type Culture Collection (No. HB 8784). The Cellfree(tm) IL2R test ELISA kit was generously provided by T-Cell Sciences for the estimation of IL2 receptor. R239 is a rabbit polyclonal antibody raised against a peptide corresponding to amino acids 169–186 of the human IL2 receptor (Rusk et al., 1988) and was a generous gift of Dr. Richard Robb (Dupont Merck Pharmaceutical Company, Wilmington, DE). HMT 3.2 is specific for the CD3 γ chain and was a generous gift from Dr. Ralph Kubo (Cytel Corporation, San Diego, CA). J31 is a polyclonal rabbit antibody raised against the COOH-terminal 13 amino acids of the human CD3 δ subunit (Alarcon et al., 1988). SP34 is specific for CD3 ϵ (Pessano et al., 1985). IC7 is an mAb raised against a peptide (DGNEEMGGI) with a sequence identical to the NH_2 terminus of the human CD3 ϵ subunit, IF10, and 2F4 are mAbs raised against a peptide with a sequence (RKGQRDLYSGLNQRRI) identical to the COOH terminus of the human CD3 ϵ subunit.

Metabolic Labeling, Immunoprecipitation, and Electrophoresis

Transfected cells were labeled metabolically with [^{35}C]methionine and

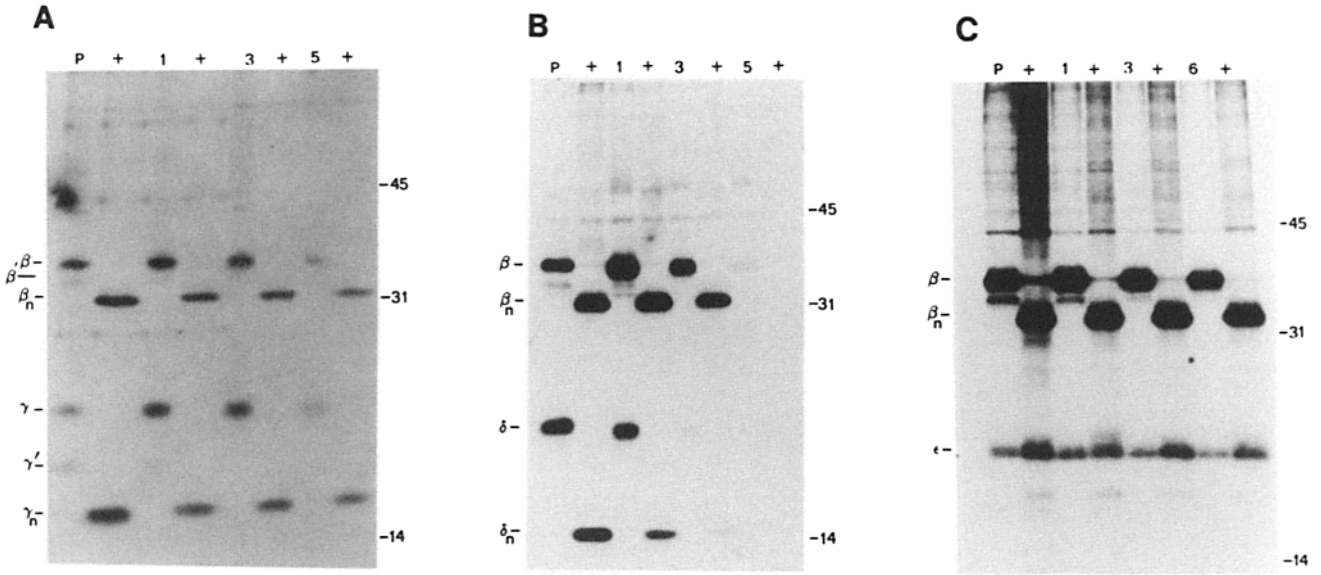


Figure 1. The degradation of the TCR β chain is slowed after assembly with CD3 subunits. CHO cells expressing the TCR β chain were transfected with cDNAs encoding with CD3 γ, δ, or ε. Cells were pulse labeled with [^{35}S]methionine/cysteine for 30 min at 37°C and then chased in complete growth media for the indicated hourly time intervals. Labeled cells were lysed in digitonin–triton immunoprecipitation buffer and precipitated with antibodies specific for the expressed CD3 subunit. Half of each precipitate was digested with endo H (+ lanes) before analysis by 12.5% SDS-PAGE under reducing conditions. The migration of molecular mass standards (in kD) is shown. (*A*) CHO cells expressing β and CD3 γ were immunoprecipitated using HMT 3.2. (*B*) CHO cells expressing β and CD3 δ were analyzed using the rabbit polyclonal antibody J31. (*C*) CHO cells expressing β and ε were immunoprecipitated using SP34. (β_n, γ_n, and δ_n indicate the deglycosylated forms of the proteins, γ′ indicates CD3 γ with one N-linked oligosaccharide.)

[^{35}S]cysteine using Trans ^{35}S-label (ICN Radiochemicals, Cleveland, OH). Cells (90% confluent 10-cm dish) were preincubated for 15 min at 37°C in methionine- and cysteine-free medium, washed, and then pulse-labeled for 30 min at 37°C in the same medium containing 0.2 mCi/ml ^{35}S. Cells were washed and then chased with complete medium for the indicated time periods. Labeled cells were lysed at 4°C in 1% NP-40 or digitonin/triton buffer (50% saturated digitonin containing 0.125% Triton X-100; see Alarcon et al., 1988), in immunoprecipitation buffer (10 mM Tris, pH 7.8, 0.15 M NaCl, 10 mM iodoacetamide, 1 mM EDTA, 1 mM PMSF, and 1 μg/ml of leupeptin, pepstatin, chymostatin, and antipain) and immunoprecipitated as described previously (Wileman et al., 1990*a*). Immunoprecipitates were washed extensively in lysis buffer and solubilized in reducing sample buffer in preparation for SDS-PAGE. PAGE was performed using 8 or 12.5% slab gels. Gels were fixed and incubated with 1 M salicylic acid before autoradiography. To ensure that excess antibody had been used for the pulse-chase immunoprecipitation analysis, lysates were reprecipitated with the relevant antibodies. In each case <10% of the original protein remained in the lysate.

Endoglycosidase Treatment

Endoglycosidase H (endo H) was purchased from Genzyme (Boston, MA) and digestions were run overnight at 37°C. Washed immunoprecipitates were first suspended in 10 μl 1% SDS and denatured by heating to 100°C for 3 min, and then diluted to 50 μl with 50 mM phosphate buffer, pH 6.0, containing 50 mU of enzyme.

Sucrose Density Sedimentation Analysis

COS cells expressing TCR/IL2R fusion proteins were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 2 h, washed, and lysed in 1 ml 1% NP-40 in immunoprecipitation buffer. 1 ml of the precleared lysate was applied to the top of a 10 ml 5–20% sucrose gradient (containing 0.1% NP-40, 50 mM Mes/Tris, pH 7.5, 1 mM PMSF, and 1 μg/ml of leupeptin, pepstatin, chymostatin, and antipain) layered over a 70% sucrose cushion. After centrifugation (40,000 rpm) for 16 h at 4°C in a SW-41 rotor (Beckman Instruments Inc., Palo Alto, CA), the gradient was separated into 1-ml fractions. The TCR/IL2R fusion proteins were immunoprecipitated with R329 and analyzed by SDS-PAGE under reducing conditions, or detected using the Cβ-specific ELISA (Wileman et al., 1990*b*). For analysis of VSV G protein, confluent CHO cells were infected with VSV (multiplicity of infection 5 pfu/cell). 5 h later cells were pulse labeled with [^{35}S]methionine and [^{35}S]cysteine for 15 min, lysed in 1% NP-40, and applied to the gradient described above. The distribution of the G protein was determined by 8% SDS-PAGE under reducing conditions.

Laser Scanning Densitometry

Quantitation of protein bands was performed by scanning autoradiograms with a laser densitometer (LKB Ultroscan XL). Gels were exposed to film for a period of time that ensured a linear range of scanning densitometry. A Gaussian integration method was used to estimate the intensity of bands (Gelscan XL software package LKB). The actin band was used to normalize data between different lanes and different experiments.

Results

Binding of CD3 Subunits Slows the Degradation of the TCR β Chain in the ER

We have shown previously that the TCR β chain is retained in the ER of CHO and COS cells and degraded rapidly with a half life of ~1 h (Wileman et al., 1990*a*,*b*). In the experiments that follow we analyze the stability of the TCR β assembled with single CD3 subunits. A CHO cell line expressing the TCR β chain was stably co-transfected with one of each of the cDNAs encoding CD3 γ, δ, or ε. Metabolically labeled cells were lysed using a buffer containing digitonin and 0.125% Triton X-100. This combination of mild detergents allows TCR chains to be co-precipitated with CD3 subunits (Alarcon et al., 1988). Fig. 1 *A* shows an immunoprecipitation of labeled cells expressing TCR β and CD3 γ. The HMT 3.2 mAb specific for the CD3 γ subunit, precipitated major bands at 25 and 35 kD. These corresponded to

Table I. The Presence of the TCR Ectodomain Increases the Stability of TCR/CD3 Complexes Formed in the ER

	Half-life (h)							
Co-expressed protein	None		CD3 γ		CD3 δ		CD3 ε	
Cell type	COS	CHO	COS	CHO	COS	CHO	COS	CHO
None			6	6	1	1	8	8
β_{tm}ILRsec	1	1	1	–	3*	–	not associated	
$\beta_{tm}C\beta$:ILRsec	1	1	5	–	8	–	4	–
TCR β [$\beta_{tm}C\beta$:Vβ]	1	1	6	6	1.5*	1.5*	8	8

The table summarizes the data presented in Figs. 1–9. The data show degradation half-lives in hours. The turnover of the TCR β chain, CD3 δ, and TCR-IL2R fusion proteins were determined after transient expression in COS cells, and in stably transfected CHO cells. Half-lives were calculated using a cycloheximide-chase ELISA protocol (Wileman et al., 1990*b*), and by densitometric scanning of autoradiographs. Both methods gave the result shown. The turnover of CD3 γ and ε expressed alone in cells, and the stability of TCR β associated with single CD3 subunits, were determined after transient expression in COS cells, and in stable CHO cell lines. The turnover of β_{tm}ILRsec and $\beta_{tm}C\beta$:ILRsec associated with CD3 subunits were only determined after transient co-expression in COS cells. Protein stabilities, in each case, were estimated by densitometric scanning of autoradiographs. The first value in each line, or column, records the intracellular half-lifes of the indicated protein expressed alone in cells (see also Wileman et al., 1990*a*,*b*). The stability of proteins bound to CD3 subunits can be found by cross reference of lines (co-expressed protein) with columns (CD3 subunit).

* Stabilization by CD3 δ is transient and leads to bi-phasic degradation kinetics, see Figs. 1, 6, and 9).

CD3 γ and TCR β, respectively. The appearance of both bands on the autoradiograph indicated the assembly of γ-β complexes in the CHO cells. Minor bands, representing partially glycosylated forms of γ and β, were seen at 18 (γ') and 32 kD (β'). Both forms of γ and β were sensitive to digestion with endo H (+ lanes) and migrated as single bands at 18 and 31 kD after incubation with the enzyme. The results indicated the formation of β–γ complexes in the ER. High levels of endo H-sensitive TCR β chain remained associated with CD3 γ through all time points (0–5 h). A half-life of 6 h was calculated for β associated with γ (Table I). Given that unassembled β chains were degraded with a half time of ~1 h (Wileman et al., 1990*a*,*b*; Table I), it was clear that CD3 γ provided a near sixfold protection of TCR β from proteolysis in the ER.

CHO cells expressing β and CD3 δ were analyzed using the same protocol. Cell lysates were immunoprecipitated using a rabbit polyclonal antibody (J31) raised against the COOH terminus of CD3 δ. J31 precipitated two proteins from pulse-labeled cells (Fig. 1 *B*). CD3 δ resolved as a single band of 20 kD, and the associated TCR β chain migrated at 35 kD. Association of CD3 δ with the TCR β chain produced a partial stabilization against degradation within the ER. Substantial levels of endo H-sensitive δ–β complexes were present within cell lysates taken at the 3-h time points. Even so, protection against proteolysis was transient and radiolabeled CD3 δ and TCR β were undetectable after 5 h. A more prolonged protection from degradation was observed for TCR β complexed to the CD3 ε subunit (Fig. 1 *C*). In this experiment, lysates of CHO cells expressing ε and β were immunoprecipitated using the ε-specific mAb, SP 34. CD3 ε (20 kD) and TCR β (35 kD) were seen in each lane of the autoradiogram demonstrating the association of CD3 ε with TCR β. The TCR β chain associated with ε was sensitive to endo H (+ lanes), indicating the formation of the ε–β complex in the ER. CD3 ε is not glycosylated and its migration did not change after digestion with endo H. High levels of endo H-sensitive β chain remained associated with ε throughout the 6-h chase. A half-life of ~8 h was calculated for β–ε complexes (Table I). Given that free β subunits were degraded with a half-life of 1 h it was clear that assembly with CD3 ε provided an eightfold increase in stability in the ER. Taken together the results showed that CD3 subunits were able to slow the degradation of the TCR β chain in the ER. The highest level of stability was found for complexes formed with γ or ε, while CD3 δ produced a transient protection from degradation. The same relationship between assembly and degradation was obtained when the experiments were repeated using transient co-transfection of COS cells (Table I).

Acidic Amino Acids within the Membrane-spanning Domains of the CD3 Proteins Are Essential for Their Associations with the TCR β Chain

The membrane-spanning domains of the CD3 γ, δ, and ε subunits contain acidic amino acid residues (Krissansen et al., 1986; Van-den Elsen et al., 1984; Gold et al., 1987) and the importance of these acidic amino acids for the association of the CD3 proteins with the TCR β chain was tested. The glutamic acid residue within the transmembrane domain of the CD3 γ subunit, and the corresponding aspartic acid of the δ and ε chains were changed to alanine by site directed mutagenesis. Mutant chains were designated γ°, δ°, and ε°. The modified CD3 subunits were co-expressed separately in COS cells with the TCR β chain, and their ability to form complexes was assessed by immunoprecipitation. COS cells

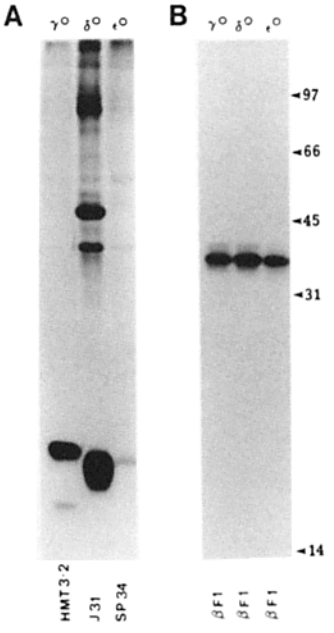


Figure 2. Acidic residues within the membrane spanning domain of the CD3 subunits are required for association with the TCR β chain. COS cells expressing the TCR β chain and either of the charge-deleted (γ°, δ°, ε°) CD3 subunits were pulse labeled for 2 h at 37°C with [^{35}S]methionine/cysteine. Cells were lysed in digitonin/triton immunoprecipitation buffer. (*A*) Half of each lysate was immunoprecipitated with antibodies specific for the expressed CD3 subunit (HMT3.2, CD3 γ°; J31, CD3 δ°, and SP34 for CD3ε°). (*B*) The remaining lysate was immunoprecipitated using the TCR β-specific mAb βF1. Precipitates were analyzed by 12.5% SDS-PAGE under reducing conditions, and analyzed by 8% SDS-PAGE under reducing conditions. The migration of molecular mass standards (in kD) is shown.

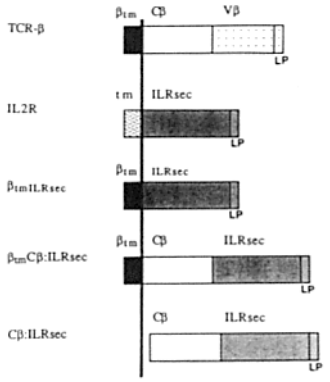


Figure 3. Schematic representation of the TCR β-IL2 receptor fusion proteins. (TCR-β) Representation indicating the relative distribution of amino acids between leader peptide (*LP*), variable region (*Vβ*), constant domain (*Cβ*), and membrane anchor (β_{tm}). (*IL2R*) The native IL2 receptor showing the distribution of amino acids between leader peptide (*LP*), extracellular domain (*ILRsec*), and membrane anchor (*tm*). β_{tm}ILRsec. The membrane anchor of the IL2 receptor was replaced by the membrane anchor of the TCR β chain (β_{tm}). (β_{tm}Cβ:ILRsec) The Vβ region and the leader peptide of the TCR β chain were replaced with the extracellular amino acids (*ILRsec*) and leader peptide (*LP*) of the IL2 receptor. (Cβ:ILRsec) Identical to β_{tm}Cβ:ILRsec, but a stop codon prevents translation of the membrane anchor of the β chain.

expressing TCR β, and the charge-deleted CD3 γ, δ, or ε subunits, were pulse labeled for 2 h and lysed in the nondisruptive digitonin/triton buffer that was used above to precipitate native TCR β-CD3 complexes (see Fig. 1). The first three lanes of Fig. 2 demonstrated that the chain-specific antibodies, HMT 3.2, J31, and SP34 precipitated the charge-deleted (γ^o, δ^o, and ϵ^o) CD3 subunits from COS cell lysates. The autoradiographs of the CD3 γ and ε immunoprecipitates were, however, completely clear in the 30–40-kD range indicating the absence of an associated β chain. Additional proteins of 37, 55, and 90 kD were seen in the J31 (δ^o) precipitation, but the identity of these proteins is not known. Fig. 2 (lanes *4–6*) show that the β-specific antibody, βF1, precipitated β from the same COS cell lysates. The results indicated that the TCR β chain was expressed in the COS cells but was unable to associate with the charge-deleted CD3 subunits. We concluded that acidic residues within the membrane anchors of CD3 subunits were required for the formation of CD3–TCR β complexes.

The Membrane Spanning Amino Acids of the TCR β Chain Contain a Structural Determinant of Degradation in the ER

In the following experiments we test whether charge-dependent interactions between membrane spanning domains can neutralize transmembrane proteolytic targeting information. In the first section we follow the stability of complexes formed when sites of subunit binding are restricted to the membrane spanning amino acids. In the second experiment we examine the role played by the Cβ domain in stabilizing interactions between transmembrane domains. The two constructs used for these experiments are compared schematically in Fig. 3. In the first, β_{tm}ILRsec, the membrane anchor (β_{tm}) of the TCR β chain was joined to the ectodomain of the IL2 receptor (ILRsec). Here the membrane anchor of the β chain serves as the only site for assembly with CD3 subunits. The second fusion protein, β_{tm}Cβ:ILRsec, includes the constant domain (Cβ), of the TCR β chain. This protein provides the combined membrane spanning (β_{tm}), and the 150 amino acids of the Cβ domain, for interaction with CD3 subunits.

The ability of the membrane anchor (β_{tm}) of the TCR β chain to mark these proteins for ER degradation was first

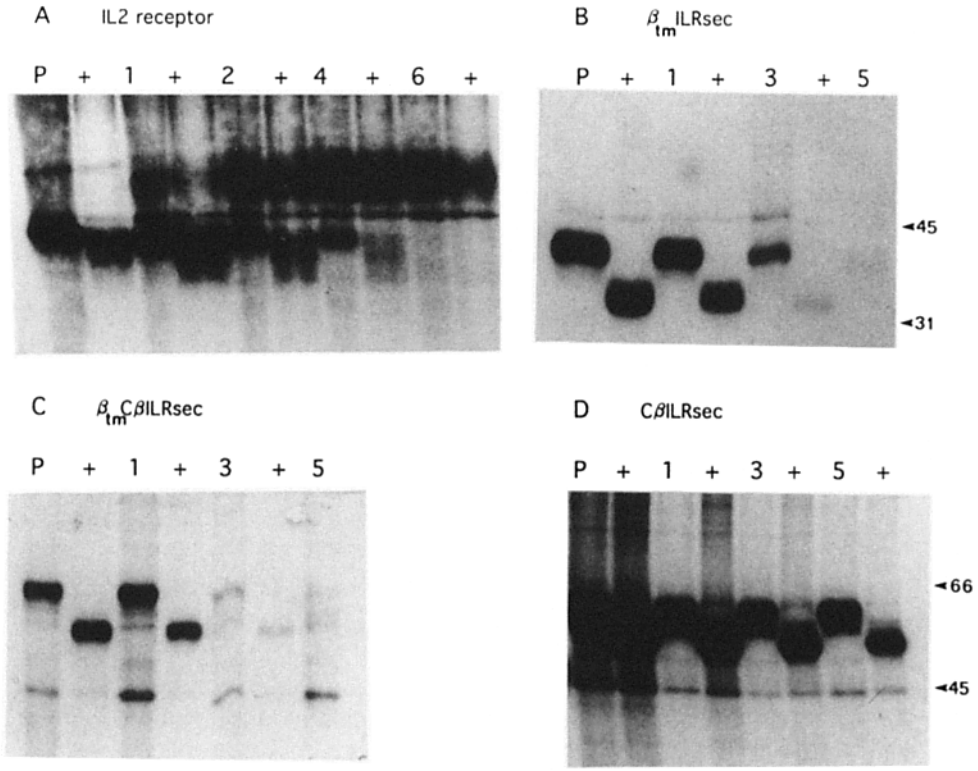


Figure 4. The membrane anchor of the TCR β chain contains a determinant of retention and degradation in the ER. COS cells expressing the native IL2 receptor or the TCRβ/IL2 receptor fusion proteins were pulse labeled with [^{35}S]methionine/cysteine for 30 min at 37°C and then chased in complete growth media for the indicated hourly time intervals. After lysis in 1% NP-40 the expressed proteins were immunoprecipitated with the IL2 receptor-specific mAb, 7G7. Half of each precipitate was digested with endo H (+) lanes. Precipitated proteins were analyzed by 12.5% SDS-PAGE under reducing conditions. The migration of molecular mass standards (in kD) is shown.

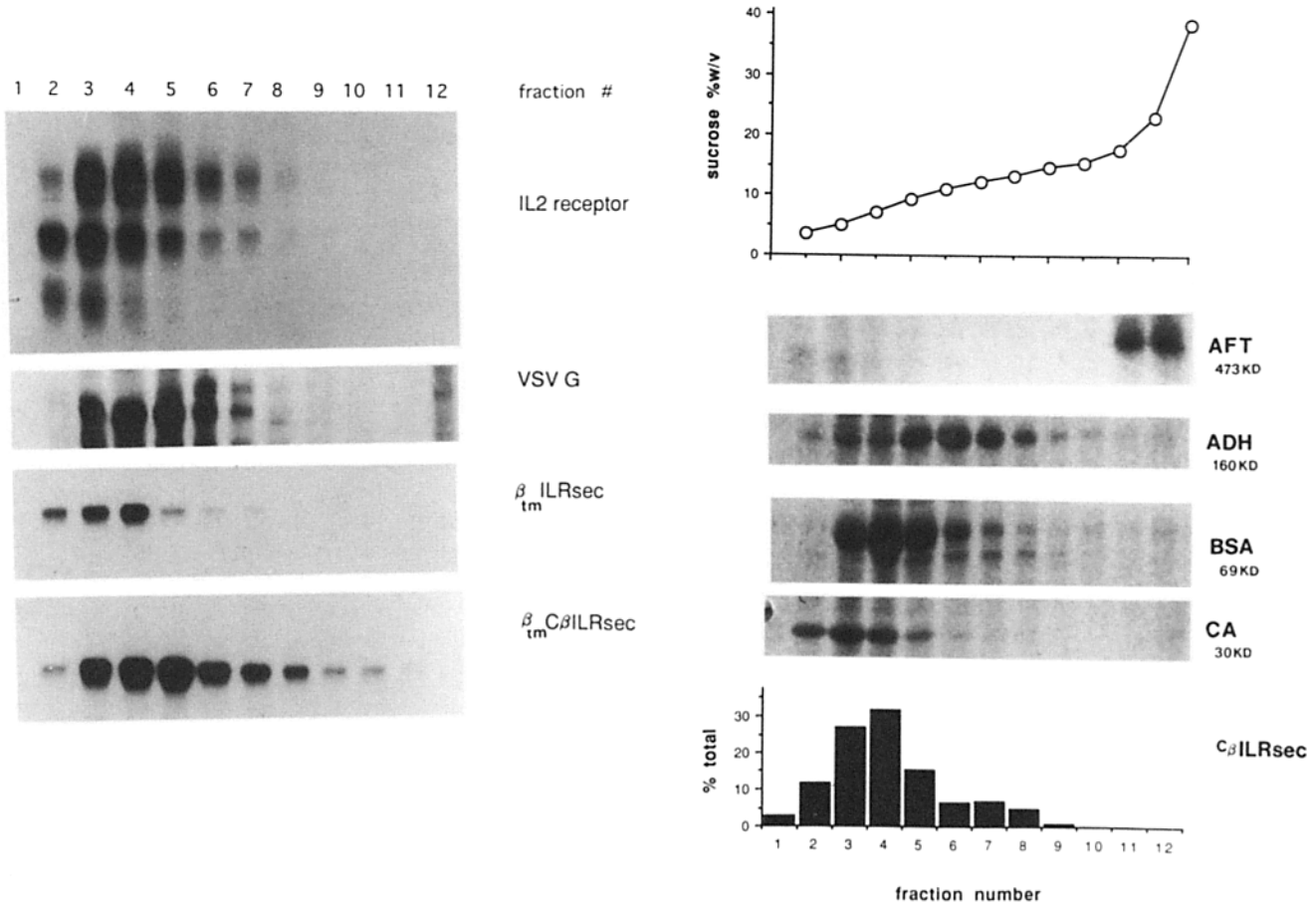


Figure 5. Sucrose density sedimentation of TCRβ-IL2R fusion proteins. COS cells expressing the native IL2 receptor or either of the β_{tm}ILRsec, $\beta_{tm}C\beta$:ILRsec, or Cβ:ILRsec fusion proteins were pulse labeled for 2 h at 37°C with [^{35}S]methionine/cysteine. Labeled cells were lysed in 1% NP-40 and fractionated by velocity centrifugation on a 5–20% sucrose gradient. The left panel compares the sedimentation of the native IL2 receptor, the β_{tm}ILRsec and $\beta_{tm}C\beta$:ILRsec fusion proteins with the migration of monomeric VSV G protein. Fractions containing IL2 receptor constructs were immunoprecipitated using a conformation-independent polyclonal antibody (R239) raised against a peptide sequence of the IL2 receptor. Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions. The right panel compares the migration of the Cβ:ILRsec fusion protein, estimated using a TCR β-specific ELISA, with the sedimentation of soluble marker proteins (*CA*, carbonic anhydrase; *BSA*, bovine serum albumin; *ADH*, alcohol dehydrogenase; *AFT*, apoferritin).

verified by COS cell transfection. COS cells expressing the native IL2 receptor, or either of the fusion proteins, were pulse labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min and chased in complete growth medium for the indicated hourly time intervals. Cell lysates (Fig. 4) were immunoprecipitated with a mAb specific for the IL2 receptor (7G7) and the extent of processing of the N-linked oligosaccharides determined by endo H digestion (+ lanes). The native IL2 receptor (Fig. 4 *A*) increased in size from 40 to 55 kD during the metabolic chase. The molecular size increase resulted from addition of N- and O-linked sugars (Trieger et al., 1986). The N-linked oligosaccharides became resistant to endo H during the chase showing that they had been modified by enzymes present within the Golgi apparatus. Fig. 4 *B* shows a similar analysis of the β_{tm}ILRsec fusion protein. Unlike the native IL2 receptor, the fusion protein failed to become resistant to endo H and was degraded during the chase. Fig. 4 *C* follows the turnover of the $\beta_{tm}C\beta$:ILRsec fusion protein. The protein was also degraded rapidly without processing of N-linked oligosaccharides, indicating degradation in the ER.

A third fusion protein, Cβ:ILRsec, was prepared by using a stop codon to prevent the translation of the membrane spanning amino acids. This protein was used as a control to show that the membrane spanning region of the β chain caused the ER degradation of the $\beta_{tm}C\beta$:ILRsec fusion protein. Fig. 4 *D* shows that the "anchor minus" Cβ:ILRsec fusion protein turned over slowly in COS cells with a half-life of ~8 h. The Cβ:ILRsec construct was not secreted during the chase and, on the basis of prolonged sensitivity to endo H, was retained in the ER. The observation that removal of the membrane anchor from the $\beta_{tm}C\beta$:ILRsec fusion protein resulted in a loss of susceptibility to degradation strongly suggested that the membrane spanning domain of the TCR β chain, and not other structural features, such as those caused by fusion of Cβ and IL2 receptor amino acids, resulted in ER degradation of the $\beta_{tm}C\beta$:ILRsec chimera.

The TCR β-IL2R Fusion Proteins Do Not Aggregate in the ER

The potential aggregation of the TCR β-ILR fusion proteins within the ER was analyzed by sucrose density centrifugation. COS cells expressing the native IL2 receptor or the

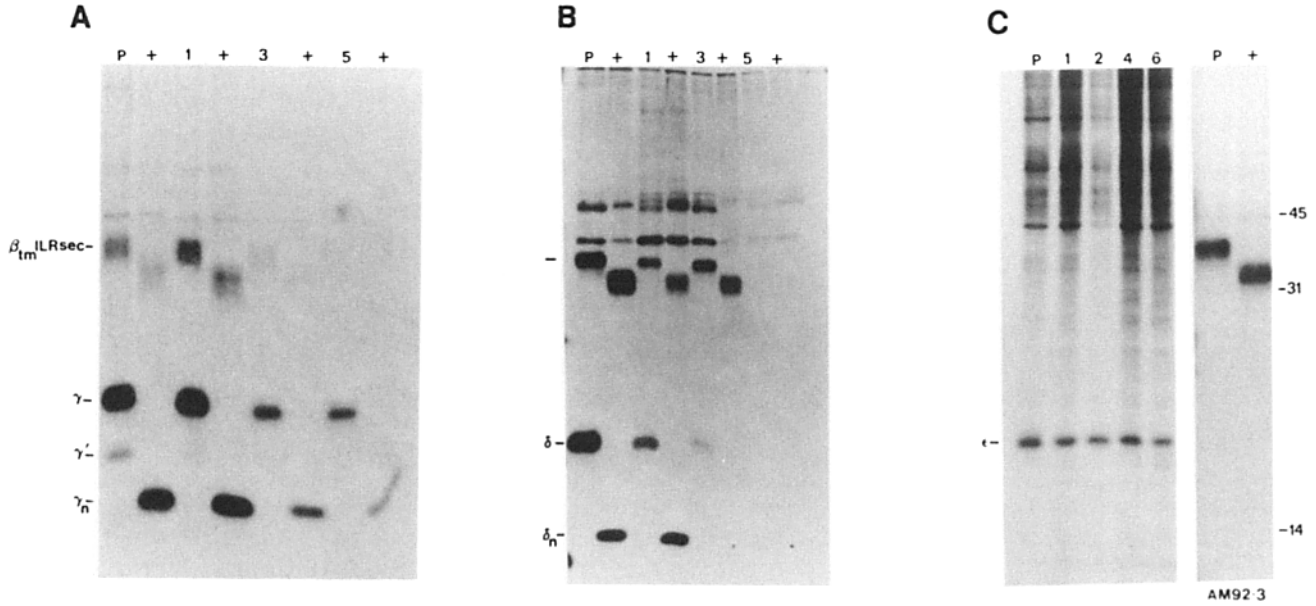


Figure 6. Associations between the CD3 subunits and the membrane anchor of the TCR β chain are unstable within the ER. COS cells expressing the β_{tm}ILRsec fusion protein and either the CD3 γ, δ, or ε subunit were pulse labeled with [^{35}S]methionine/cysteine for 30 min at 37°C and then chased in complete growth media for the indicated hourly time intervals. Labeled cells were lysed in digitonin–triton immunoprecipitation buffer and precipitated with antibodies specific for the expressed CD3 subunit. Half of each precipitate was digested with endo H (+ lanes) before analysis by 12.5% SDS-PAGE under reducing conditions. The migration of molecular mass standards (in kD) is shown. (*A*) COS cells expressing β_{tm}ILRsec and CD3 γ were immunoprecipitated using HMT 3.2. (*B*) COS cells expressing β_{tm}ILRsec and CD3 δ were analyzed using the rabbit polyclonal antibody J31. (*C*) COS cells expressing β_{tm}ILRsec and CD3 ε were immunoprecipitated using SP34, or the IL2 receptor-specific mAb AM92.3 (*right lanes*). (γ_n and δ_n indicate the deglycosylated forms of the proteins, γ′ indicates CD3 γ with one N-linked oligosaccharide).

TCR β-IL2R fusion proteins were pulse labeled for 2 h, lysed in 1% NP-40, and layered over a 5–20% sucrose gradient. After centrifugation for 16 h at 4°C gradient fractions were immunoprecipitated with a rabbit polyclonal antibody, R239, that binds to both denatured and native IL2 receptor (Rusk et al., 1988). This ensured the precipitation of native and potentially misfolded or aggregated material from the gradient. The sucrose gradients were analyzed with reference to the monomeric form of VSV G protein, an integral membrane protein of 60 kD.

In the left hand panel of Fig. 5 the sedimentation characteristics of VSV G protein, the native IL2 receptor, and the membrane bound β_{tm}ILRsec and β_{tm}Cβ:ILRsec fusion proteins were compared. The β_{tm}ILRsec and β_{tm}Cβ:IL2Rsec proteins resolved at 40 and 60 kD, respectively, when analyzed by SDS-PAGE (Fig. 4, *B* and *C*), and monomeric forms of these proteins would be expected to migrate on sucrose gradients within the range covered by IL2 receptor and 60-kD VSV G protein. Peak levels of β_{tm}ILRsec were precipitated from fractions 2, 3, and 4, the same fractions that contained the newly synthesized 40-kD IL2 receptor. The β_{tm}Cβ:ILRsec protein concentrated in slightly heavier fractions (3, 4, and 5) that contained monomeric VSV G protein (60 kD), and the 55 kD fully glycosylated form of the native IL2 receptor. On the basis of this analysis we concluded that the membrane-anchored TCR β-IL2R fusion proteins migrated predominantly as monomers on the gradients and did not aggregate within the ER. In the right panel, sucrose gradients were calibrated using soluble proteins, and the migration of the "anchor minus" Cβ:ILRsec construct across the gradient was assessed using a TCR Cβ-specific ELISA. Peak levels of Cβ:ILRsec were recovered in fractions containing the 69-kD marker (BSA). The results again argued against aggregation of the 60-kD "anchor minus" control protein within the lumen of the ER.

Associations between the CD3 γ, δ, and ε Subunits, and the Membrane-spanning Amino Acids of the TCR β Chain, Are Unstable within the ER

The ability of CD3 subunits to bind to the membrane spanning amino acids of the TCR β chain, and produce stable complexes in the ER, was assessed by expressing single CD3 subunits, and the β_{tm}ILRsec protein, in COS cells. Cells were pulse labeled for 30 min with [^{35}S]methionine and [^{35}S]cysteine, and chased in complete media containing cycloheximide, to stop new protein synthesis, for up to 5 h. Cells were then lysed in the nondisruptive digitonin/triton buffer and immunoprecipitated with antibodies specific for CD3 subunits. The quantity of fusion protein associated with CD3 subunits at each time point was determined by SDS-PAGE analysis of immunoprecipitates. The level of unbound fusion protein remaining in cell lysates after each immunoprecipitation were determined using an IL2 receptor-specific ELISA. Since the addition of cycloheximide to the chase media prevented new protein synthesis, the loss of ELISA signal gave an assessment of the turnover of the unbound fusion protein during the experiment.

Fig. 6 *A* shows an autoradiograph obtained when labeled COS cells expressing CD3 γ and the β_{tm}ILRsec protein were immunoprecipitated using the γ-specific mAb, HMT 3.2. The antibody precipitated the 18- and 25-kD forms of

Table II. The Ectodomain of the TCR β Chain Promotes Assembly with CD3 Subunits

	Percent bound to CD3 subunits		
Co-expressed protein	CD3 γ	CD3 δ	CD3 ε
β_{tm}ILRsec	43	52	0
β_{tm}Cβ:ILRsec	56	65	34
TCR β [β_{tm}Cβ:Vβ]	60	63	78

COS cells co-expressing individual CD3 γ, δ or ε subunits and the native TCR β, or the indicated TCR-IL2R fusion protein, were immunoprecipitated with CD3-specific antibodies. The distribution of the native TCR β or the indicated TCR-IL2R fusion protein between the supernatant and the immunoprecipitate was assessed using Cβ or IL2 receptor-specific ELISAs. The results record the % of the expressed protein that was depleted from cell lysates after immunoprecipitation of CD3 subunits.

CD3 γ, and also the β_{tm}ILRsec chimera, seen clearly above γ at 40 kD. The results showed that CD3 γ associated with the membrane-spanning amino acids of the TCR β chain. Both CD3 γ and the fusion protein were endo H sensitive, indicating formation of a complex within the ER. The CD3 γ specific antibody removed ~40% of the IL2 receptor-specific ELISA signal from cell lysates (Table II) indicating that nearly half of the β_{tm}ILRsec fusion protein synthesized by the cells was complexed to γ. Association of the chimera with CD3 γ was, however, short-lived, and the endo H–sensitive 40-kD band was lost from immunoprecipitates between 1 and 3 h after labeling. In Fig. 7 *A* densitometric scanning of autoradiographs was used to assess the stability of the β_{tm}ILRsec chimera bound to CD3 γ. The graph indicated that, after a lag period of 1 h, the fusion protein was lost from immunoprecipitates with a half-life of ~60 min. The ELISA was used to follow the stability of unbound fusion protein during the course of the experiment. The ELISA signal remaining in lysates after immunoprecipitated, also shown in Fig. 7 *A*, fell during the metabolic chase indicating the degradation of the uncomplexed protein. Taken together the experiments demonstrated that complexes formed between CD3 γ and the β_{tm}ILRsec protein were unstable within the ER and were not protected from degradation.

Figs. 6 *B* and 7 *B* show a similar experiment following the association of CD3 δ with the β_{tm}ILRsec fusion protein. Lysates prepared from COS cells expressing both proteins were immunoprecipitated with the δ-specific antibody, J31. J31 depleted ~50% of the synthesized fusion protein from cell lysates (Table II). The immunoprecipitated proteins resolved at 20 kD (δ) and 40 kD (β_{tm}ILRsec) and were sensitive to endo H. The results indicated that the complex formed with δ was assembled within the ER. Three hours into the chase, ~90% of the pulse-labeled CD3 δ–β_{tm}ILRsec complex could be immunoprecipitated from cell lysates. This initial stabilization against degradation was, however, transient and neither δ nor the fusion protein remained after 5 h. Fig. 7 *B* compares the stability of the bound and unbound fusion protein during the experiment. The ELISA measure of the unbound fusion protein was reduced by 90% at the 3-h time point indicating the rapid degradation of unassociated β_{tm}ILRsec. Importantly, the material lost from immunoprecipitates between the 3- and 5-h time points could not be detected in the supernatants. We conclude from these results that the β_{tm}ILRsec protein dissociated from δ between the 3- and 5-h time points and was degraded.

The third co-expression study analyzed interactions between the CD3 ε and the β_{tm}ILRsec protein (Fig. 6 *C*). SP34 precipitated the 20 kD CD3 ε subunit from lysates prepared from pulse-labeled COS cells. High levels of the β_{tm}ILRsec protein were precipitated from the same lysates using the IL2 receptor specific antibody, AM 92.3 (right hand lanes). The SP34 immunoprecipitations were, however, clear in the 30–40-kD range, suggesting that complexes formed between ε and the β_{tm}ILRsec protein were unstable. This was supported by the observation that SP34 was unable to deplete the COS cell lysate of IL2 receptor ELISA signal (Table II). In the autoradiograph shown in Fig. 8, a panel of antibodies binding to different epitopes within CD3 ε were used in an attempt to co-precipitate ε and the β_{tm}ILRsec fusion protein. IC7 is an anti-peptide mAb that binds to the NH_2 terminus of CD3 ε; IF10 and 2F4 are anti-peptide mAbs that bind to the COOH terminus of ε. The left panel showed that each of the antibodies was able to precipitate native ε–β complexes, these antibodies were, however, unable to co-precipitate ε and the β_{tm}ILRsec protein (right panel).

The levels of CD3 ε precipitated at each time point remained constant throughout the 6 h of the experiment but there was no evidence for an association with the 30–40-kD β_{tm}ILRsec protein with ε at later time points. These results argued against a slow rate of complex formation between these two proteins. In fact, Fig. 7 *C* showed that 80% of the unbound β_{tm}ILRsec fusion protein was lost from cell lysates within 2 h of synthesis, indicating its rapid degradation. The rate of degradation of the fusion protein in cells co-expressing ε was the same as that determined for the β_{tm}ILRsec protein expressed alone in cells (Fig. 4 *B*). Taken together the results indicated that the CD3 ε subunit was unable to associate with the fusion protein and stabilize against degradation in the ER.

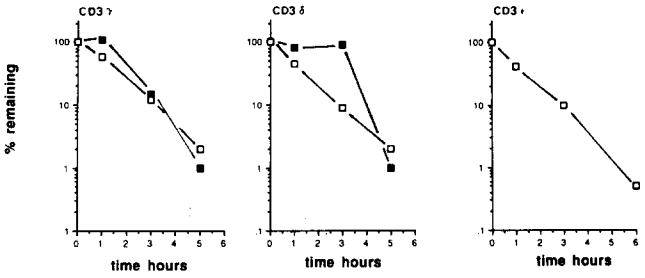


Figure 7. Both the bound and unbound forms of β_{tm}ILRsec protein are unstable in COS cells expressing CD3 subunits. COS cells expressing the β_{tm}ILRsec protein and either the CD3 γ, δ, or ε subunit were pulse labeled for 30 min at 37°C using [^{35}S]methionine/cysteine. Washed cells were than warmed to 37°C in culture media containing 10 μg/ml cycloheximide. At the indicated time points cells were lysed in digitonin/triton buffer and CD3-β_{tm}ILRsec complexes absorbed using antibodies to the expressed CD3 subunit (HMT 3.2 for CD3 γ; J31 for CD3 δ and SP34 for CD3 ε). The relative levels of β_{tm}ILRsec bound to CD3 subunits was determined at each time point from SDS PAGE analysis of immunoprecipitates (see representative autoradiograms in Figure 6) and densitometric scanning of autoradiograms (■). The quantity of unbound β_{tm}ILRsec remaining in the supernatants was determined using an IL2 receptor-specific ELISA (□).

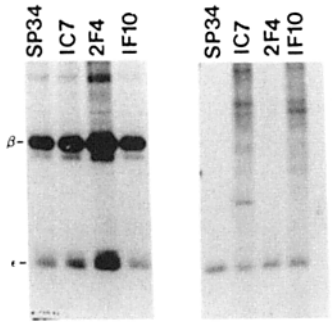


Figure 8. Native ϵ–β complexes, but not ϵ–β_{tm}ILRsec are stable to detergent lysis and immunoprecipitation by panel of ϵ-specific mAbs. COS cells expressing CD3 ϵ and either the native TCR β chain (*left*) or the β_{tm}ILRsec fusion protein (*right*) were pulse labeled with [^{35}S]methionine/cysteine for 2 h at 37°C. Labeled cells were lysed in digitonin–triton immunoprecipitation buffer and precipitated with the indicated mAbs (SP34, IC7, 2F4, IF10) specific for CD3 ϵ. Immunoprecipitates were analyzed by 12.5% SDS-PAGE under reducing conditions.

CD3 Subunits Form Stable Associations with the Combined Membrane-spanning and Constant Domains of the TCR β Chain

The ability of the first 150 amino acids of the constant domain of the TCR β chain to stabilize complexes was tested by co-expressing CD3 subunits with the $\beta_{tm}C\beta$:ILRsec protein. In Fig. 9 *A*, labeled COS cells expressing CD3 γ and the $\beta_{tm}C\beta$:ILRsec chimera were immunoprecipitated with HMT 3.2. The antibody precipitated endo H–sensitive forms of CD3 γ and the 60-kD $\beta_{tm}C\beta$:ILRsec fusion protein and removed 56% of the $\beta_{tm}C\beta$:ILRsec from cell lysates (Table II). Significantly, high levels of endo H–sensitive $\beta_{tm}C\beta$:ILRsec protein remained associated with CD3 γ throughout the 5-h chase. The half-life of the $\beta_{tm}C\beta$:ILRsec protein associated with CD3 γ was 6 h (Table I), sixfold longer than complexes formed when membrane spanning domains formed the only sites for subunit assembly (Fig. 6 *A*).

The stabilizing influence of the Cβ domain on assembly with CD3 δ was assessed similarly in the next experiment. J31 precipitated CD3 δ, and the associated 60-kD fusion protein (Fig. 9 *B*). High levels of endo H–sensitive $\beta_{tm}C\beta$:ILRsec chimera survived throughout the 5 h of the experiment. The complex containing CD3 δ and the $\beta_{tm}C\beta$:ILRsec chimera survived with a half-life of ~8 h. Given that association with CD3 δ was transient when binding sites were restricted to membrane spanning domains (Fig. 6 *C*) the results again showed that the presence of the Cβ domain markedly increased stability of complexes within the ER.

The possibility that the Cβ domains may similarly enhance binding to CD3 ϵ, and promote the formation of stable complexes within the ER was also tested. After metabolic labeling, COS cells expressing CD3 ϵ and the $\beta_{tm}C\beta$:ILRsec were lysed, and immunoprecipitated with the ϵ-specific mAb, SP34 (Fig. 9 *C*). CD3 ϵ associated with the 60-kD fusion protein and a significant level of endo H–sensitive fusion protein remained bound to CD3 ϵ throughout the 5-h metabolic chase. Given that we were unable to find any evidence for an association of the ϵ subunit with the β_{tm}ILRsec protein (Figs. 6 and 8) we conclude that the Cβ domain stabilizes associations with CD3 ϵ. The 4-h half-life of the

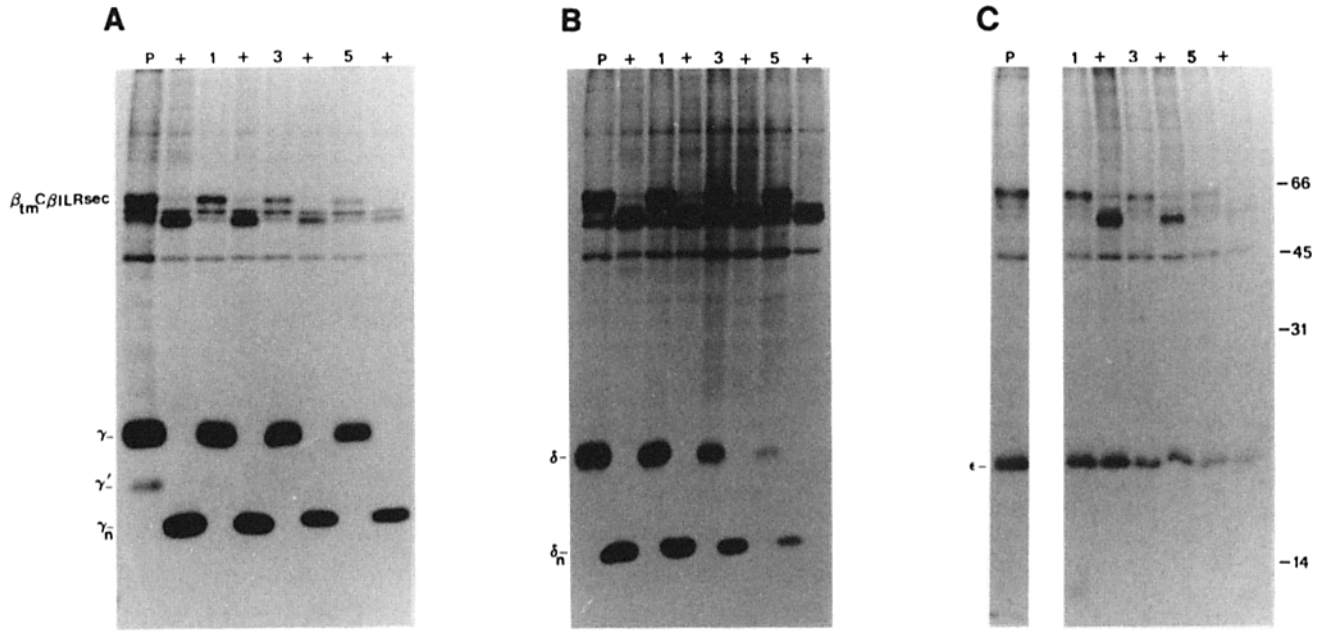


Figure 9. The constant domain of the TCR β chain promotes assembly with CD3 subunits, and increases stability within the ER. COS cells transfected with the $\beta_{tm}C\beta$:ILRsec fusion protein and the indicated CD3 subunit, were pulse labeled with [^{35}S]methionine/cysteine for 30 min at 37°C, and then chased in complete growth media for the indicated hourly time intervals. Labeled cells were lysed in digitonin–triton immunoprecipitation buffer and precipitated with antibodies specific for the expressed CD3 subunit. Half of each precipitate was digested with endo H (+ lanes) before analysis by 12.5% SDS-PAGE under reducing conditions. The migration of molecular mass standards (in kD) is shown. (*A*) COS cells expressing $\beta_{tm}C\beta$:ILRsec and CD3 γ were immunoprecipitated using HMT 3.2. (*B*) COS cells expressing $\beta_{tm}C\beta$:ILRsec and CD3 δ were analyzed using the rabbit polyclonal antibody J31. (*C*) COS cells expressing $\beta_{tm}C\beta$:ILRsec and CD3 ϵ were immunoprecipitated using SP34 (γ_n and δ_n indicate the deglycosylated forms of the proteins, γ' indicates CD3 γ with one N-linked oligosaccharide).

ϵ–β_{tm}Cβ:ILRsec complex was less than the 8-h half-life recorded for the native β chain stabilized by CD3 ϵ (Table I). The native β chain contains Vβ and Cβ domains and the greater stability of native ϵ–β complexes may reflect a stability arising from interactions between CD3 ϵ and Vβ amino acids. The contribution made by Vβ residues was also reflected in the levels of protein co-precipitated with CD3 ϵ. Higher levels of native β chain (78%) were co-precipitated with ϵ (Fig. 1 *C*; Table II) when compared with the amount of β_{tm}Cβ:ILRsec fusion protein (34%) (Fig. 9 *C*; Table II).

Discussion

One of the most interesting features concerning the biosynthesis of the TCR is the observation that receptor is protected from the action of proteases while it assembles in the ER (Wileman et al., 1990*a*; Bonifacino et al., 1989). These observations imply that structural features within the receptor chains that render them sensitive to ER degradation become masked during receptor assembly. In the case of the TCR α and β chains, structural determinants of ER degradation have been mapped to membrane spanning domains (Wileman et al., 1990*c*; Bonifacino et al., 1990*a*,*b*, 1991). In this present study we ask whether the binding of single CD3 γ, δ, or ϵ subunits to this localized determinant prevents proteolysis within the ER. Co-expression experiments showed that single CD3 subunits were, indeed, able to slow the proteolysis of the native TCR β chain in the ER. The highest level of stability for β was observed for binding to single CD3 γ or ϵ subunits. This process raised the half-life of the β chain from 1 h to greater than 6 h. The CD3 δ subunit produced a transient increase in stability. δ–β complexes survived for 3 h, but were eventually lost from cells between 3 and 5 h of synthesis. The results implied that CD3 subunits were able to mask proteolytic targeting information. The lower stability of β–δ complexes may result because CD3 δ is sensitive to ER proteases (Wileman et al., 1990*a*,*b*). The ϵ–β and γ–β complexes may survive longer, because the CD3 subunit in each case is more resistant than δ, to ER proteolysis (Table I).

The structural basis for the assembly and stabilization of TCR–CD3 complexes in the ER was examined further. Unusually for integral membrane proteins, the membrane spanning domains of the CD3 proteins contain single acidic amino acids (Van den Elsen et al., 1984; Gold et al., 1986; Krissansen et al., 1986). The importance of these charged residues for association with the TCR β chain was assessed by changing them to alanine. Significantly, the charge-deleted CD3 subunits were unable to bind to the TCR β chain. Several studies have demonstrated that basic residues within the membrane-spanning domains of TCR α and β chains are also necessary for receptor assembly (Morley et al., 1988; Alcover et al., 1990; John et al., 1989; Blumberg et al., 1990). Our reciprocal experiments using charge-deleted CD3 subunits reported here support the proposal that TCR-CD3 assembly requires the neutralization of oppositely charged membrane-spanning residues within the lipid bilayer (Cosson et al., 1991; Green, 1991).

In the next experiments we took advantage of the β_{tm}ILRsec fusion protein and asked whether the direct binding of CD3 subunits to the membrane spanning domain of the TCR β chain would mask proteolytic targeting information. The membrane anchor of the TCR β chain (β_{tm}) marked the β_{tm}ILRsec fusion protein for degradation within the ER, and at the same time, offered membrane-spanning residues as the only site for assembly with CD3 subunits. Single CD3 γ or CD3 δ subunits bound efficiently to the fusion protein, even so, complexes containing γ or δ were short-lived within the ER. The binding of CD3 ϵ to the β_{tm}ILRsec protein appeared even weaker. The transmembrane domain of CD3 ϵ contained an aspartic acid that was essential for the formation of native ϵ–β complexes, but we were unable to find any evidence for the binding of ϵ to the β_{tm}ILRsec fusion protein.

It was clear from these results that the stability of complexes within the ER was reduced when sites of assembly were restricted to membrane spanning residues. These results prompted us to assess the influence of the Cβ domain on the protection of complexes from ER degradation. The presence of the Cβ domain in the fusion protein markedly increased the stability of complexes formed in the ER. All three CD3 subunits bound to the β_{tm}Cβ:ILRsec fusion protein and the complexes survived for several hours. Assembly with CD3 γ produced a level of stability ($t_{1/2}$ = 6 h) comparable to that found for γ–β complexes. The Cβ domain could not fully complement the high stability of the ϵ–β pair ($t_{1/2}$ = 8 h) and ϵ–β_{tm}Cβ:ILRsec complexes survived with a half life of only 4 h. One possible explanation for this observation could be that interactions with the Vβ amino acids are also important for the ability of ϵ to stabilize the β chain. The Vβ domain certainly enhanced the efficiency of assembly of ϵ with co-expressed proteins. Only 40% of the synthesized β_{tm}Cβ:ILRsec chimera, which lacked Vβ, could be immunoprecipitated with ϵ, whereas this value increased to 78% for the native β chain. Although important for assembly with ϵ, we found little evidence for a similar effect of Vβ amino acids on assembly with CD3 γ or δ. The quantity of β_{tm}Cβ:ILRsec and native β chain associated with γ and δ were approximately equal (Table II).

Above we made the assumption that fusion proteins can be used to determine the domains of the TCR β chain that are important for assembly with CD3 subunits. Several studies have demonstrated that some fusion proteins fold incorrectly and aggregate after synthesis (Adams and Rose, 1986; Gething et al., 1986; reviewed in Rose and Doms, 1988; Hurtley and Helenius, 1989). It was possible that several of our conclusions may be invalid if the fusion proteins used in this study were misfolded and aggregated within the ER. In particular we (Wileman et al., 1990*c*), and others (Bonifacino et al., 1990*a*,*b*, 1991), have suggested that the specific structural features within the membrane spanning domains of the TCR α and β chains, rather than aggregation, mark TCR α and β chains for rapid turnover in the ER. Sucrose density sedimentation analysis was used to assess the level of aggregation of the β_{tm}ILRsec, β_{tm}Cβ:ILRsec, and Cβ:ILRsec proteins. The bulk of each protein migrated on gradients at a position expected of a monomer. This physical criteria argued against aggregation in the ER. This conclusion was supported by our ability to immunoprecipitate each fusion protein with the conformation-dependent antibody, 7G7 (Fig. 5). 7G7 recognizes a cysteine-dependent epitope that is lost when any one of 10 of the 12 extracellular cysteine residues of the IL2 receptor are modified by mutation (Rusk et al., 1988). The epitope recognized by 7G7 retained its correct conformation in each of the TCR-IL2R fusion proteins (Fig.

4). Within the limitations of these experiments we could find little evidence for gross misfolding or aggregation of the fusion proteins within the ER. We think, therefore, that their relative ability to bind to CD3 proteins, and be stabilized against ER proteolysis, reflects the physiological role played by β_{tm} and Cβ domains during TCR assembly.

It is not known how the binding of CD3 subunits to TCR chains leads to stability within the ER. It has been proposed that positively charged residues within membrane-spanning regions of TCR chains may be recognized directly by the proteolytic apparatus of the ER (reviewed in Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991). In this model, the neutralization of charged transmembrane residues that drives heterodimer assembly, may simultaneously neutralize the signals within the transmembrane domains that mark proteins for degradation in the ER (Bonifacino et al., 1990*b*; Manolios et al., 1990; Green, 1991; Cosson et al., 1991). Our data confirm the importance of charged transmembrane residues for the binding of CD3 subunits to the TCR β chain, but the results show clear differences in the stability of complexes formed in the ER. Interactions with CD3 subunits that were restricted to membrane spanning domains were, for example, short lived within the ER. The presence of Cβ amino acids stabilized complexes and markedly slowed their turnover in the ER. The results show that the stability of complexes in the ER can be influenced by structural features that are separate from the charged residues in membrane-spanning regions. Interactions between ectodomains within the lumen of the ER may promote subunit assembly and slow subunit dissociation. Such a mechanism would prolong the masking of transmembrane residues from direct recognition by the proteolytic machinery of the ER and thereby increase the stability of complexes in the ER.

Interestingly, Fig. 1 provides indirect evidence suggesting that the lumenal domain of CD3 δ is degraded before proteolysis of the δ–β complex. In *B*, the levels of CD3 δ fall dramatically between the 1 and 3 h time points, yet the levels of co-precipitated β chain remain relatively constant. If the lumenal domain of CD3 δ were degraded this would remove all the methionine and cysteine residues from the protein, and reduce the size of δ to ~8 kD. Both processes would cause the observed loss of radiolabeled δ from the autoradiograph. Significantly, the immunoprecipitation was carried out using an antibody that bound to the cytoplasmic tail of CD3 δ. The observed immunoprecipitation of β, in the apparent absence of δ, could be explained if proteolysis had not progressed into the membrane-spanning domain. Under such circumstances the site for assembly with β would remain intact, allowing co-precipitation of β with the antibody directed against the cytoplasmic tail of CD3 δ. A similar effect was seen for CD3 δ associated with the fusion proteins (Fig. 6 *B* and 9 *B*). If this interpretation is correct, then interactions between membrane-spanning domains mask proteolytic targeting information within the β chain, but not CD3 δ. Proteolysis of the lumenal domain of δ leads to destabilization of the complex and loss of β between the 3 and 5 h time points.

While we show a marked effect of the Cβ domain on protein stability within the ER, we were unable to provide direct evidence for binding of CD3 subunits to Cβ residues. CD3 subunits failed to bind to the native TCR β chain for example, when charged membrane-spanning residues were changed to alanine. In this respect the assembly of the TCR differs from the assembly of the nicotinic acetylcholine receptor or the trimerization of influenza hemagglutinin within the ER. The NH_2-terminal amino acids of the α, γ, and δ subunits of the acetylcholine receptor form specific complexes within the ER lumen, in the absence of membrane-spanning domains (Yu and Hall, 1991; Verrall and Hall, 1992; Hall, 1992). Furthermore, anchor minus forms of influenza hemagglutinin assemble into transport-competent trimers in the ER, and are secreted by cells (Singh et al., 1990).

Our results suggest that the assembly of the entire $\alpha\beta\gamma\delta\epsilon\zeta$-$\zeta$ TCR complex within the ER takes place through a hierarchy of domain interactions. The TCR β chain has only one basic (lysine) residue in a position that could interact with the membrane-spanning acidic residues of CD3 subunits (Cosson et al., 1991). It seems unlikely that CD3 γ, δ, and ϵ subunits would bind to this lysine residue simultaneously. In the ER of T cells, the CD3 subunits may compete with one another for associations with newly synthesized β chains. A process of subunit exclusion may give rise to the T cell antigen receptors containing either CD3 γ or CD3 δ that have been identified recently on the surface of T cells (Alarcon et al., 1991). The CD3 subunits associate with one another in the absence of TCR α and β chains (Wileman et al., 1990*a*; Bonifacino et al., 1989) and this leads to stabilization of CD3 δ. Since charge-deleted CD3 subunits form γ–ϵ and δ–ϵ complexes (Hall et al., 1991; Manolios et al., 1991), these interactions do not require charged membrane-spanning residues. Associations between extracellular and cytoplasmic domains may predominate for the formation of stable CD3 complexes and leave the acidic membrane spanning residues of CD3 subunits available for binding to TCR chains. Interestingly, not all subunit interactions lead to the masking of proteolytic targeting information. This is most apparent for complexes formed between proteins that are themselves substrates for ER proteolysis. For example, murine TCR α–β (Bonifacino et al., 1990*b*) and human CD3 δ–TCR α and CD3 δ–TCR β complexes (Wileman et al., 1990*a*; Hall et al., 1991) assemble in the ER, but are degraded shortly afterwards. Given the complexity of TCR assembly, and the diversity of subunit interactions, such models remain incomplete. It nevertheless seems likely that the structures of the TCR chains and the CD3 subunits have evolved together to ensure that proper interactions between transmembrane and ectodomains promote receptor assembly and protect the TCR from degradation in the ER. This in turn allows correctly assembled receptors to survive for transport to the cell surface.

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