

The γ and ϵ Subunits of the CD3 Complex Inhibit Pre-Golgi Degradation of Newly Synthesized T Cell Antigen Receptors

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Abstract. The T cell receptor for antigen (TCR) is composed of six different transmembrane proteins. T cells carefully control the intracellular transport of the receptor and allow only complete receptors to reach the plasma membrane. In an attempt to understand how T cells regulate this process, we used c-DNA transfection and subunit-specific antibodies to follow the intracellular transport of five subunits ($\alpha\beta\gamma\delta\epsilon$) of the receptor. In particular, we assessed the intracellular stability of each chain. Our results showed that the chains were markedly different in their susceptibility to intracellular degradation. TCR α and β and CD3 δ were degraded rapidly, whereas CD3 γ and ϵ were stable. An analysis of the N-linked oligosaccharides of the glycoprotein subunits suggested that the chains were unable to reach the medial Golgi during the metabolic chase. This was supported by immunofluorescence micrographs that showed both the stable CD3 γ and unstable CD3 δ chain localized in the endoplasmic reticulum. To study the effects of

subunit associations on intracellular transport we used cotransfection to reconstitute precise combinations of subunits. Associations between stable and unstable subunits expressed in the same cell led to the formation of stable complexes. These complexes were retained in or close to the endoplasmic reticulum. The results suggested that the intracellular transport of the T cell receptor could be regulated by two mechanisms. The TCR α and β and CD3 δ subunits were degraded rapidly and as a consequence failed to reach the plasma membrane. CD3 γ or ϵ were stable but were retained inside the cell. The results also demonstrated that there was an interplay between the two pathways such that the CD3 γ and ϵ subunits were able to protect labile chains from rapid intracellular degradation. In this way, they could seed subunit assembly in or close to the endoplasmic reticulum and allow a stable receptor to form before its transport to the plasma membrane.

MANY cell surface receptors are composed of more than one polypeptide chain and to function efficiently their subunits have to be assembled correctly (Carlin and Merlie, 1986). The T cell receptor for antigen (TCR)¹ is structurally one of the more complex cell surface receptors and is assembled from at least six transmembrane proteins (Clevers et al., 1988; Oettgen et al., 1986; Samelson et al., 1985). The receptor has two variable chains, α and β (or γ and δ) linked by a disulfide bond; these chains recognize antigens presented to the T cell and have a structure similar to immunoglobulin. The other four chains ($\gamma\delta\epsilon\zeta$) are invariant. The strong structural homologies between γ and δ (Krissansen et al., 1986), and the observation that the genes for γ , δ , and ϵ are closely linked (Evans et al., 1988; Tunnacliffe et al., 1988) has led to these three subunits being referred to as the CD3 complex (Wiessman et al., 1988). The ζ chain is unrelated structurally or genetically to

the CD3 proteins and associates with the receptor as a disulfide linked homodimer (Oettgen et al., 1986; Samelson et al., 1985; Weissman et al., 1988).

The TCR has to be transported to the surface of a T cell before it can function in the complex processes of antigen recognition. Many studies have shown that only fully assembled receptors reach the plasma membrane and have suggested that the transport of the receptor within the T cell is regulated carefully. Evidence for such a mechanism has come primarily from studies of T cell mutants that fail to synthesize one or more of the chains of the receptor (Ohashi et al., 1985; Weiss and Stobo, 1984; Saito et al., 1987). These T cells will assemble partial receptor complexes but they will not transport them efficiently to the cell surface unless the missing chain is replaced by cDNA transfection (Ohashi et al., 1985; Weiss and Stobo, 1984; Saito et al., 1987).

In murine T cells, the subunits of the receptor appear to be synthesized in excess over the number that are finally incorporated into the plasma membrane (Minami et al., 1987). An intracellular pool of subunits results and they turn

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1. *Abbreviations used in this paper:* Endo-H, endoglycosidase-H; TCR, T cell receptor.

over rapidly. It has been proposed that this susceptibility to rapid intracellular degradation is one method used by T cells to prevent the surface expression of partial receptor complexes. In this study, we have sought to determine how T cell receptors can escape rapid intracellular degradation and reach the plasma membrane intact. Evidence that they can be protected from degradation has come from earlier studies on T cells that lack either the TCR α or β chains (Chen et al., 1988; Alarcon et al., 1988), or CD3 δ (Bonifacino et al., 1989). These cells assemble partial complexes that are unable to reach the cell surface but significantly, the complexes are stable within the cell. These observations suggested that some partial complexes can be retained in an early compartment of the secretory pathway in a conformation that is not susceptible to degradation.

The $\alpha\beta\gamma\delta$ and ϵ subunits of the receptor form a pentameric (TCR/CD3) complex that is transported efficiently from the endoplasmic reticulum to the medial Golgi of T cells (Sussman et al., 1988). In this study, we have used cDNA transfection to determine the intracellular fate of each of these five subunits and have been able to analyze the events that take place before the movement of the TCR/CD3 complex from the ER into the secretory pathway. In particular we have used subunit-specific mAbs to assess the intrinsic susceptibility of each chain to degradation and have then reconstituted partial complexes to assess the effects played by subunit assembly on the stability of the receptor.

Our results show that when expressed alone in cells, TCR α and β and CD3 δ are degraded rapidly without passing through the medial Golgi. In contrast, CD3 γ and ϵ are metabolically stable and are retained in or close to the ER. Interestingly, when combinations of chains are transfected into cells the stable CD3 γ and ϵ chains form complexes with the labile chains and markedly slow their turnover. These results imply that the CD3 γ and ϵ subunits stabilize the receptor as it assembles in preparation for its transport to the Golgi.

Materials and Methods

Cells

CHO cell lines DUX B11 (dhfr⁻ CRL 9010) and K1 (ATCC# CCI 61) were obtained from American Type Culture Collection (Rockville, MD). Unless under selection, these cells were maintained in 5% CO₂ at 37°C in a base medium of α -MEM supplemented with proline (15 μ g/ml), or RPMI supplemented with glutamine (2 mM), and 10 μ g/ml each of adenosine, thymidine and deoxyadenosine. The African green monkey kidney cell line COS-1 was a generous gift of Dr. Brian Seed (Massachusetts General Hospital, Boston, MA) and was cultured at 37°C in DME supplemented with 2 mM glutamine and buffered using 10% CO₂. All media contained 8% heat-inactivated FCS. Tissue culture media were purchased from Gibco Laboratories (Grand Island, NY) or Sigma Chemical Co. (St. Louis, MO).

Transfection

(a) **Vectors.** Standard molecular biology protocols (Maniatis et al., 1982) were used throughout. Restriction enzyme digestion was used to remove the reading frames encoding TCR α and β chains (derived from the T cell line Jurkat) from the plasmids pY14 and pYT35, respectively (Yanagi et al., 1984). The reading frames encoding the CD3 subunits were excised from the following plasmids. CD3 γ from pT3 γ -3 (Krissansen et al., 1986), CD3 δ from pPGBC9 (Van den Elsen et al., 1984), and CD3 ϵ from pDJ4 (Gold et al., 1986). The coding sequences were subcloned into a mammalian expression vector containing a transcriptional unit constructed from the adenovirus major late promoter. Resistance genes, xanthine-guanine phosphoribosyltransferase (*gpt*) (Mulligan and Berg, 1981), APH(3')II phos-

photransferase (*neo*) (Southern and Berg, 1982) or dihydrofolate reductase (*dhfr*) (Kaufmann and Sharp, 1982), driven by the SV-40 early promoter, were linked in tandem to the transcriptional unit. In brief, the following constructions were made to allow for the selection of stably transfected and cotransfected cell lines. TCR α -(*gpt*), TCR β -(*gpt*), CD3 δ -(*neo*), CD3 γ -(*dhfr* or *neo*), CD3 ϵ -(*dhfr* or *neo*).

(b) **Selection.** CHO cells were transfected using the calcium phosphate precipitation procedure (Graham and Van der Eb, 1973). CHO K1 cells transfected with the *gpt* gene constructs were selected in growth media supplemented with hypoxanthine (135 μ g/ml), xanthine (13 μ g/ml), and mycophenolic acid (10 μ g/ml, Gibco Laboratories) while those transfected with the *neo* gene were selected in base media containing 500 μ g/ml G418 (Gibco Laboratories). CHO DUX B11 cells were transfected using the *dhfr* gene constructs and were selected using α -MEM lacking ribonucleosides and deoxyribonucleosides supplemented with 8% dialyzed FCS and 15 μ g/ml proline. Resistant cell lines were cloned by limiting dilution and Northern blotting was used to select cells expressing highest levels of transfected TCR or CD3 genes. Reconstitution of multiple subunits within cells was performed by sequential transfection and recloning. Transfectants carrying two selection markers were cultured in appropriate media. (*neo/dhfr*: α -MEM lacking ribonucleosides and deoxyribonucleosides supplemented with 8% dialyzed FCS, 15 μ g/ml proline, and 1 mg/ml G418. *gpt/neo*: base media supplemented with 135 μ g/ml hypoxanthine, 13 μ g/ml xanthine, 10 μ g/ml mycophenolic acid, and 500 μ g/ml G418.) The combinations of selection markers used to generate the cell lines expressing multiple subunits were as follows. $\alpha\beta\epsilon$ and $\alpha\beta\delta$: α/β (both *gpt*) cells were transfected with either ϵ (*neo*) or δ (*neo*). $\alpha\beta\gamma\delta$, $\alpha\beta\delta\epsilon$, and $\alpha\beta\gamma\epsilon$: in separate experiments, α/β (both *gpt*) cells were transfected with both γ and δ (*dhfr* and *neo*, respectively) or δ and ϵ (*neo* and *dhfr*, respectively) or γ and ϵ (*dhfr* and *neo*). $\delta\epsilon$: CD3 δ cells (*dhfr*) were transfected with CD3 δ (*neo*). $\gamma\delta\epsilon$: CHO DUXB11 cells were transfected with CD3 ϵ and γ (both *dhfr*) and δ (*neo*).

Antibodies. The subunit-specific mAbs used and their specificities were as follows. IdentiT α α -Fl, TCR α ; IdentiT β β -Fl, TCR β ; HMT 3.2 (kindly provided by Dr. Ralph Kubo, National Jewish Center for Immunology and Respiratory Disease, Denver, CO), CD3 γ , SP64, CD3 γ and δ ; and SP34, CD3 ϵ . The specificities of the CD3 specific SP series of mouse mAbs have been described previously (Pessano et al., 1985). The TCR-specific antibodies, α -Fl and β -Fl were provided by T Cell Sciences (Cambridge, MA). Second antibodies and fluorescein and biotin-conjugated second antibodies were purchased from Cappel Laboratories (Malvern, PA).

Immunofluorescence. Monkey COS cells were transfected transiently with CD3 γ or CD3 δ as follows. Restriction enzyme digestion was used to excise the reading frames encoding CD3 γ and CD3 δ from the plasmids pT3 γ -3 and pPGBC9, respectively, and subclone them separately into the COS cell expression vector PMNC8 (Seed and Aruffo, 1988). COS cells at 60% confluence on a 10-cm dish were transfected by 4 h incubation at 37°C with 2 μ g vector DNA dissolved in 5 ml DME containing 10% NuSerum (Collaborative Research, New Bedford, MA), 400 μ g/ml DEAE dextran (Pharmacia Fine Chemicals, Piscataway, NJ) and 100 μ M chloroquine. Washed cells were then shocked by 2 min incubation at room temperature in DME containing 10% DMSO. After overnight culture in DME supplemented with 5% heat-inactivated FCS, cells were trypsinized, allowed to adhere to sterile cover slips, and cultured as before. The next day they were washed in PBS, fixed using 4% paraformaldehyde/0.2% glutaraldehyde, and permeabilized using 0.1% Triton X-100 followed by 70% ethanol. Expressed protein was visualized using subunit-specific antibodies. Cells transfected with CD3 γ were incubated with HMT 3.2, washed to remove excess antibody and then incubated with biotinylated goat anti-hamster IgG followed by fluorescein-conjugated avidin (Becton Dickinson & Co., Mountain View, CA). Cells expressing CD3 δ were incubated with SP64, washed and then incubated with fluorescein-conjugated goat anti-mouse IgG. Oligosaccharides processed to contain *N*-acetyl glucosamine and/or sialic acid were visualized in the same cells using wheat germ agglutinin conjugated to Texas Red (EY Labs, San Mateo, CA).

Metabolic Labeling, Immunoprecipitation, and Electrophoresis

Transfected cells were labeled metabolically with [³⁵S]methionine and [³⁵S]cysteine using Trans ³⁵S-label (ICN Radiochemicals, Cleveland, OH). Cells (90% confluent 10-cm dish) were preincubated for 1 h 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 ³⁵S. Cells were washed and then chased with complete medium for the indicated time periods. Labeled cells were lysed at 4°C in immunoprecipitation buffer (10

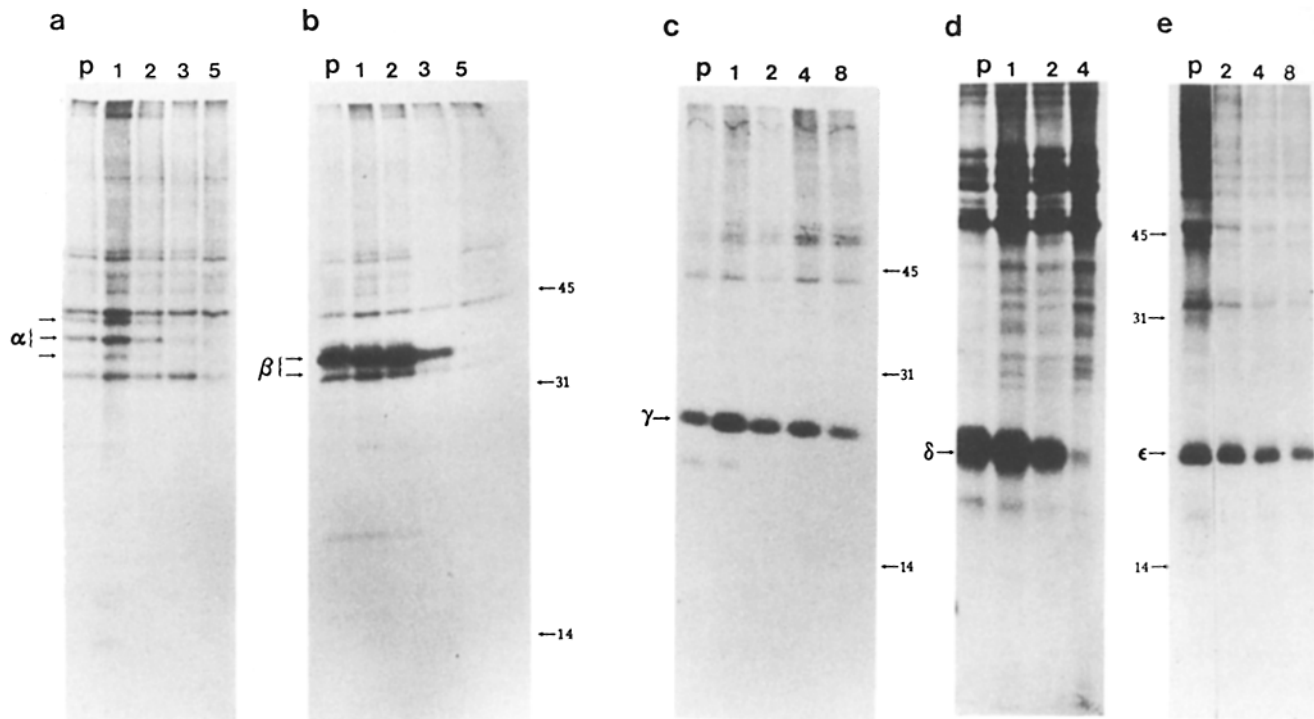


Figure 1. Differential stability of the subunits of the TCR/CD3 complex. CHO cells were transfected stably with the α , β , δ , or ϵ subunits of the receptor. COS cells were transfected transiently with the γ chain. Cells were pulse-labeled with [35 S]methionine/cysteine for 30 min at 37°C and then chased in complete media for the indicated hourly time intervals. After lysis in 1% NP-40, the transfected proteins were immunoprecipitated with subunit-specific antibodies and analyzed by 12.5% SDS-PAGE under reducing conditions. The positions of molecular mass markers (kilodaltons) are shown. (a) The transfected TCR α (gpt) subunit was precipitated by mAb α -F1. The positions of the glycosylated forms of α are indicated. (b) The transfected TCR β (gpt) was precipitated by β -F1. The positions of the glycosylated forms of β are indicated. (c) COS cells were transfected with CD3 γ and precipitated with HMT 3.2. (d) The transfected CD3 δ chain (neo) was precipitated with SP64. (e) The transfected CD3 ϵ chain (dhfr) was precipitated with SP34.

mM triethanolamine, pH 7.8, 0.15 M NaCl, 10 mM iodoacetamide, 1 mM EDTA, 1 mM PMSF and 1 μ g/ml of the following peptide protease inhibitors: leupeptin, pepstatin, chymostatin, and antipain) containing either 1% NP-40, for analysis of single chains, or 50% saturated digitonin for the analysis of subunit interactions (Alarcon et al., 1988; Oettgen et al., 1986). Lysates were clarified by centrifugation, precleared, and immunoprecipitated as described previously (Alarcon et al., 1988). Preformed immune complexes were prepared from the mouse mAbs using goat anti-mouse IgG (Cappel Laboratories), and Protein A-Sepharose was used to immobilize the hamster mAb HMT 3.2. Immunoprecipitates were washed extensively in immunoprecipitation buffer and solubilized in reducing sample buffer in preparation for SDS-PAGE. PAGE was performed using 12.5% slab gels (Laemmli, 1970). 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

Endoglycosidases were purchased from Genzyme Corp. (Boston, MA) and digestions were run overnight at 37°C. For digestion with endoglycosidase H (Endo-H), washed immunoprecipitates were first suspended in 10 μ l 1% SDS and denatured by heating to 100°C for 3 min and then diluted to 50 μ l with 50 mM phosphate buffer, pH 6.0 containing 50 mU of Endo-H. For digestion with endoglycosidase-F (Endo-F) washed immunoprecipitates were denatured by boiling in 7.5 μ l 0.5% SDS in 0.1 M 2-mercaptoethanol, after which 15 μ l of 0.25 M sodium phosphate buffer, pH 8.6, containing 1 mM EDTA and 1% NP-40 and 50 mU Endo-F were added.

Laser Scanning Densitometry

Quantitation of protein bands was performed by scanning autoradiograms

with a laser densitometer (Ultrascan XL; LKB Instruments, Gaithersburg, MD). A Gaussian integration method was used to estimate the intensity of bands (Gelscan XL software package; LKB Instruments). The actin band was used to normalize data between different lanes and different experiments.

Results

Intrinsic Stability of the Subunits of the TCR/CD3 Complex

CHO cells were transfected stably with either the α , β , δ or ϵ subunits of the TCR/CD3 complex, whereas CD3 γ was expressed transiently in monkey COS cells. Cells were analyzed by pulse-chase immunoprecipitation using mAbs specific for the transfected subunit. Analysis of pulse-labeled cells (first lane in each panel of Fig. 1), showed that the cells produced proteins with characteristics expected of each transfected subunit. TCR α was visualized as three precipitable forms of ~40 kD (a), that migrated between an upper actin band seen at 45 kD and a lower nonspecific band seen at 30 kD. TCR β resolved as two precipitating proteins at 35 kD (b). These bands represented the different glycosylated forms of the proteins and they migrate with the same molecular weights as TCR α and β chains precipitated from human T cells (Alarcon et al., 1988; Koning et al., 1988; Oettgen et al., 1986). CD3 δ and ϵ were precipitated as proteins of 20 kD (d and e), whereas CD3 γ was slightly larger at 23–25

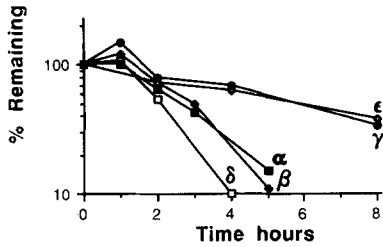


Figure 2. Survival of individual TCR/CD3 subunits determined by scanning densitometric analysis of autoradiograms. Pulse-chase immunoprecipitation of each chain were carried out as described in the legend to Fig. 1. Precipitated protein was quantified by densitometric scanning of autoradiograms. The quantity of the subunits remaining at each time point was expressed as a percentage of the protein precipitated from pulse-labeled cells. The data points represent the average of at least two estimations for each chain.

kD (c). When the pulse-labeled cells were chased the relative stabilities of the subunits became evident. The quantity of immunoprecipitable TCR α and β and CD3 δ remained constant during the first 2 h after the pulse label. After this lag period they were degraded rapidly. TCR α and β and CD3 δ had similar intracellular half lives during the degradation phase estimated by scanning densitometry of autoradiograms to be ~ 1 –1.5 h (Fig. 2). In contrast, CD3 γ and ϵ were more stable and the gels show the quantity of protein precipitated during a prolonged metabolic chase. Significant levels of both chains survived intact 8 h after the pulse label and their half lives calculated from Fig. 2 were between 6 and 8 h.

Our evidence for degradation is based on the disappearance of immunoprecipitable material from cell lysates during the metabolic chase. An alternative explanation would be that the subunits of the receptor changed conformation during the chase period such that they were no longer recog-

nized by the mAbs. Two lines of evidence made this seem unlikely. First, all of the antibodies used in the study immunoprecipitated their respective antigens from the plasma membrane of radioiodinated T cells (Alarcon et al., 1988). This illustrated that epitopes were not lost due to conformational changes taking place during the assembly and transport of the subunits to the cell surface. Second, the same relative stability of subunits was recorded when experiments were repeated using antibodies specific for different epitopes (data not shown). In total we used three monoclonal antibodies to the β chain (β F1, WT76, and C305 [Weiss and Stobo, 1984]) and a rabbit polyclonal antisera, H38 (Fabbi et al., 1985); two mAbs were used to study CD3 γ (SP 64, HMT 3.2), CD3 δ (SP64, SP19), and CD3 ϵ (SP 34, SP6). Four of these antibodies (β F1, SP 64, SP 34, and HMT 3.2) were used successfully in Western blotting experiments and therefore recognized epitopes present in the fully denatured peptides (Pessano et al., 1985; data not shown).

To see how far along the secretory pathway the chains were transported, the pulse-chase immunoprecipitations were repeated for the glycoprotein subunits. At each time point precipitates were digested with Endo-H to assess the glycosylation state of their attached sugars. Fig. 3 shows that the molecular weight of the precipitated proteins fell as a consequence of digestion. The three glycosylated forms of TCR α (a), and the two forms of TCR β (b) migrated as single bands of 32 kD, whereas CD3 γ (c) and δ (d) were reduced to molecular weights of 14 and 16 kD, respectively. At no time point during the chase did Endo-H-resistant forms of the TCR α and β and CD3 δ chains appear and this implied that they were degraded without passing through the medial Golgi. The bulk of the γ chain remained sensitive to Endo-H during the chase, nevertheless a small quantity of Endo-H resistant material migrating at 18 kD was detected. This protein migrated at 16 kD when digested with Endo-F (see Fig. 8), showing that it was indeed a glycosylated form of CD3 γ . The

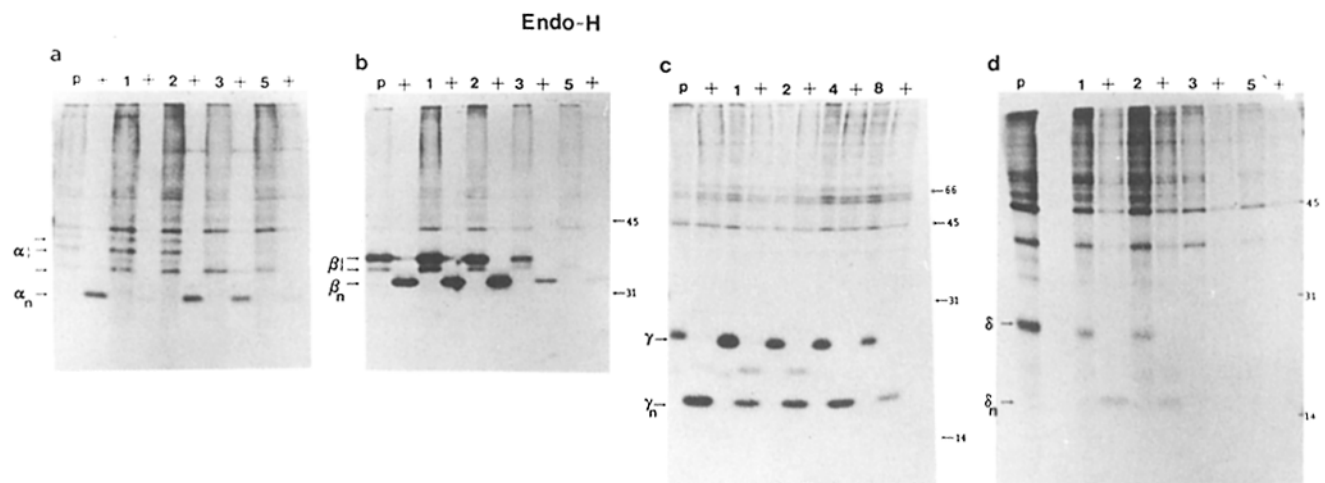


Figure 3. The individual subunits of the receptor fail to reach the medial Golgi. The transfected cell lines described in the legend to Fig. 1 were pulse-labeled with [35 S]methionine/cysteine for 30 min at 37°C and chased in complete medium for the indicated hourly time intervals. Half the precipitated protein for each time point was digested with Endo-H (+). The samples were analyzed using 12.5% SDS/PAGE under reducing conditions. The positions of the molecular mass markers (kilodaltons) are shown. (a) TCR α was precipitated using 3A8, the positions of the glycosylated (α) and deglycosylated (α_n) forms of α are indicated. (b) TCR β was precipitated using β F-1, the positions of the glycosylated (β) and deglycosylated (β_n) chains are shown. (c) CD3 γ was precipitated with HMT 3.2, the position of the glycosylated and deglycosylated (γ_n) chain is indicated. (d) The transfected δ chain was precipitated using SP64. The position of the deglycosylated δ chain is indicated (δ_n).

resistance of this form of γ to Endo-H suggested that a small fraction of CD3 γ could reach the medial Golgi, the bulk of the chain was nevertheless retained in an early compartment of the secretory pathway. CD3 ϵ does not have *N*-linked oligosaccharides and was not tested.

Immunofluorescence analysis was used to further define the intracellular structures involved in the transport of the subunits of the receptor. By virtue of the antibodies available we were able to study the steady-state distribution of the stable CD3 γ chain and the labile CD3 δ subunit. These proteins were expressed separately in monkey COS cells. Fixed cells were permeabilized, incubated with subunit-specific antibodies, and then visualized using fluorescently labeled second antibodies. In parallel experiments, oligosaccharides processed in the Golgi to forms containing *N*-acetyl glucosamine and sialic acid were detected by incubating cells with wheat germ agglutinin conjugated to Texas Red. Fig. 4 compares the immunofluorescence staining patterns of the two CD3 subunits, *A* shows CD3 δ and *B* shows CD3 γ . The distribution of immunofluorescence was similar for both proteins and was in agreement with the Endo-H analysis recorded above. Staining was restricted primarily to a reticular membrane network that extended from the nucleus to the periphery of the COS cell. This pattern strongly resembled the known structure of the ER of COS cells (Lee and Chen, 1988; Terasaki et al., 1984). Antibody and lectin staining patterns were compared in the same cell in the lower two panels. Antibody staining of the CD3 subunits was distinct from the diffuse and globular stain next to the nucleus visualized by wheat germ agglutinin. The cells were thicker around the nucleus and there were many superimposed layers of reticulum stained by the antibody to CD3 subunits. This resulted in intense staining in the perinuclear region and as a result, it was not possible to exclude entirely some colocalization of antibody and lectin, nonetheless the bulk distribution of the transfected CD3 subunits appeared separate from that of processed oligosaccharides.

Association and Stabilization of CD3 Proteins

In these experiments we used vectors containing different resistance markers to cotransfect CHO cells with precise combinations of subunits. The cells were labeled metabolically and then lysed using mild detergent conditions to maintain associations between chains. Immunoprecipitation with subunit-specific antibodies allowed us to assess specific interactions between subunits and see if interactions between subunits would affect their intracellular transport.

Fig. 5 shows immunoprecipitation of CHO cells cotransfected with the labile CD3 δ subunit and the stable ϵ chain. The cells were pulse-labeled for 30 min and chased for up to 8 h and then precipitated with an antibody specific for the ϵ chain (SP34; *left*) or CD3 δ (SP64, *right*). The molecular masses of ϵ and δ are the same (20 kD) but they will migrate separately from one another after removal of the *N*-linked sugars from 14-kD protein backbone of δ by digestion with Endo-F. The gel shown on the left demonstrated the association of the two subunits because the ϵ -specific antibody was able to coprecipitate δ . The gel on the right showed a reciprocal experiment where an antibody to the δ chain coprecipitated ϵ . In addition, two faint bands could be seen between the transfected chains. Control experiments using untransfected CHO cells showed that these proteins did not bind to

CD3-specific immune complexes (data not shown) and probably associated with the CD3 subunits. Their role, if any in the intracellular transport of the transfected chains was not known.

Pulse-chase analysis was used to see if δ could be stabilized through its association with ϵ . As seen in the cells transfected with ϵ alone the ϵ subunit survived throughout the 8-h chase. Significantly, the δ subunit, which was degraded rapidly when it was transfected alone (Fig. 1 *d*) appeared to be metabolically stable in these cells. Such an interpretation was possible only in the absence of a large excess of CD3 δ . If there were an excess of δ in these cells then the unbound δ available at each time point could replace the δ subunits that had dissociated from ϵ during the chase. This would give the impression of stabilization even though the dissociated δ chains had been degraded. The gel on the right showed cell lysates that were precipitated independently with antibodies to δ and demonstrated that the cells contained approximately equal quantities of each subunit. Notably, all the δ chain present in the cells was metabolically stable.

Immunoprecipitation of a CHO cell transfected with all three CD3 subunits is shown in Fig. 6. As with the δ/ϵ clones the cells were pulse-labeled for 30 min and chased for up to 8 h. Immunoprecipitation of lysates using the ϵ -specific mAb is shown on the left. To confirm the presence of γ and δ the precipitates were digested with Endo-F. Fig. 6 *A* shows that the antibody to CD3 ϵ precipitated three bands. The upper 20 kD band was ϵ and the lower two bands were γ (16 kD) and δ (14 kD). The δ and γ chains coprecipitated with ϵ suggesting that the transfected CHO cells assembled a CD3 complex. The pulse-chase immunoprecipitation protocol was used to assess the stability of the CD3 complex and gels were analyzed by scanning laser densitometry (Fig. 6 *B*). These data illustrated the marked stability of the CD3 $\gamma\delta\epsilon$ complex and showed the survival of all three subunits throughout the 8-h chase. Endo-H digestion was used to follow the glycosylation state of the CD3 complex during the chase (Fig. 6 *C*). As with the Endo-F digests three bands (γ , δ , and ϵ) were visible. A comparison of the Endo-F and Endo-H digestions suggested that the majority of the total protein precipitated, seen in the Endo-F digests, remained sensitive to Endo-H throughout the chase. The results showed that the assembled CD3 subunits were retained in an early compartment of the secretory pathway and did not reach the medial Golgi.

Association of CD3 Proteins with TCR Chains

In the following experiments we used cotransfection to investigate the possible stabilization of TCR chains through their association with subunits of the CD3 complex. To do this a TCR α/β CHO cell was cotransfected with combinations of CD3 proteins and as before, pulse-chase immunoprecipitation analysis was used to assess the extent of associations and the resulting stability of partial TCR/CD3 complexes.

Fig. 7 *a* shows analysis of the α/β cell transfected with the stable CD3 ϵ subunit. The SP34 mAb that was specific for ϵ precipitated ϵ and also TCR β from the cells but did not coprecipitate the TCR α chain. There were two bands precipitated at the molecular weight expected of β . The lower band, particularly evident after Endo-H digestion of TCR β , was judged to be nonspecific. (In control experiments it bound to SP34 immune complexes incubated with lysates

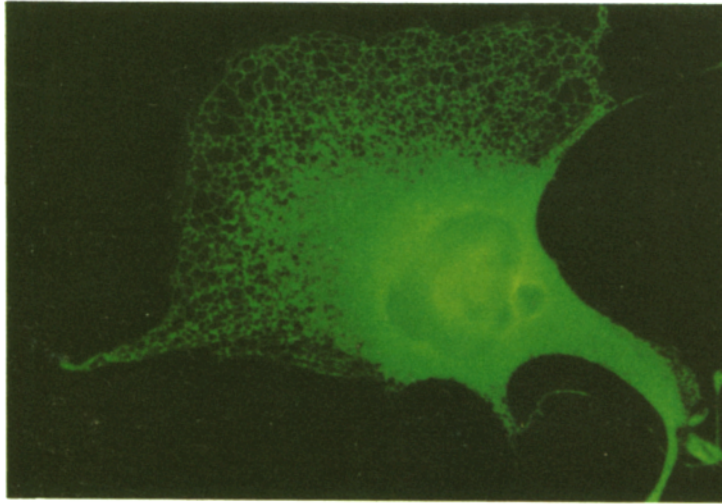
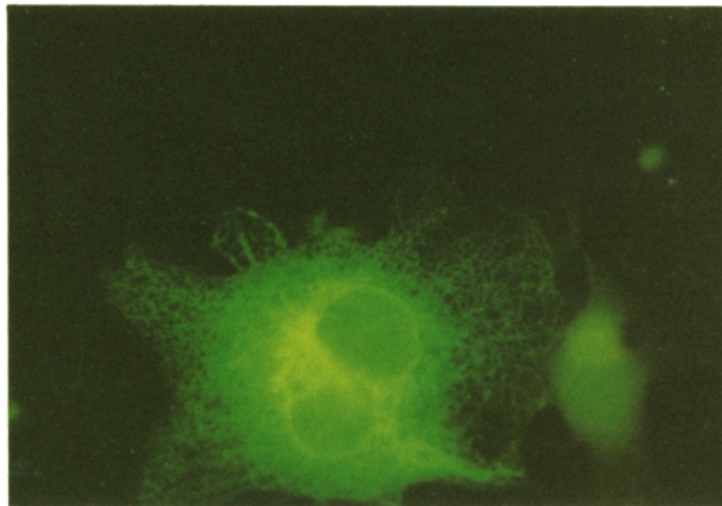
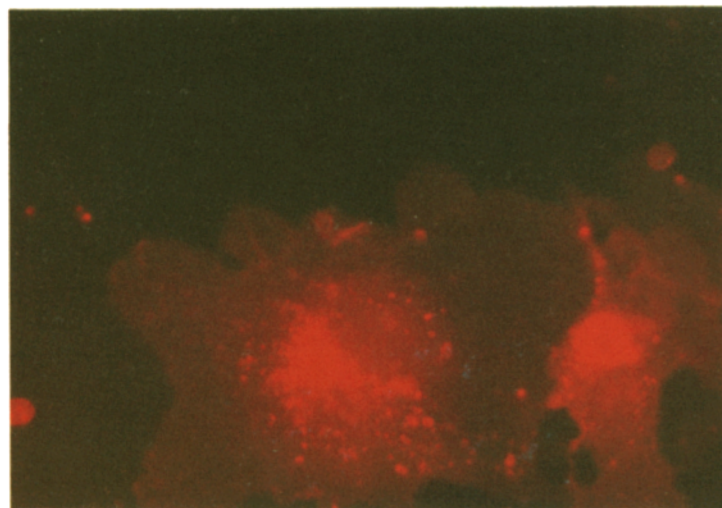
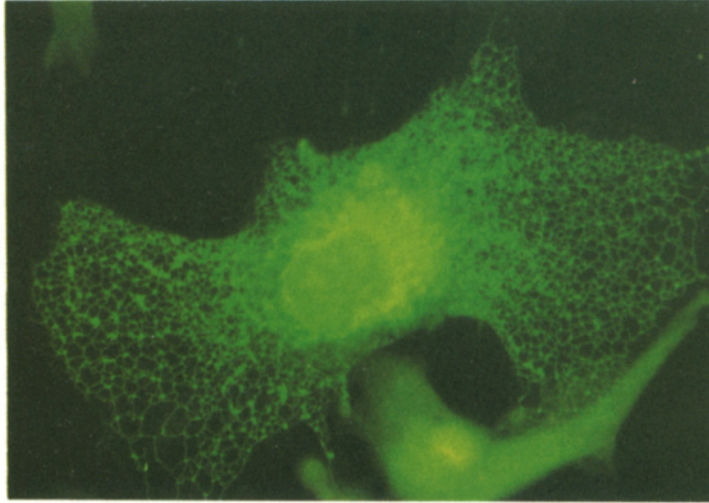
A**CD3 δ** **SP64****SP64****WGA**

Figure 4. Immunofluorescence localization of stable and degraded CD3 subunits in monkey COS cells. Monkey COS cells were transfected transiently with either CD3 γ (stable) or CD3 δ (degraded). 48 h later the cells were washed, fixed using paraformaldehyde and glutaraldehyde, and permeabilized with Triton and incubated with appropriate subunit-specific antibodies. Each micrograph shows intense fluorescence in the transfected cells, whereas untransfected cells in the same micrographs appear dull. (A) The top micrograph shows COS cells that were transfected with CD3 δ and incubated with the mAb SP 64. The antibody was visualized with fluorescein-conjugated goat anti-mouse IgG. The bottom two micrographs compare the distribution of SP 64 and Texas Red-conjugated WGA in the same cells. (B) The upper micrograph shows COS cells that were transfected with CD3 γ and incubated with the mAb HMT 3.2. The antibody was visualized using biotinylated goat anti-hamster IgG and fluorescein-conjugated avidin. The lower two micrographs compare the distribution of HMT 3.2 and Texas Red-conjugated wheat germ agglutinin in the same cells.

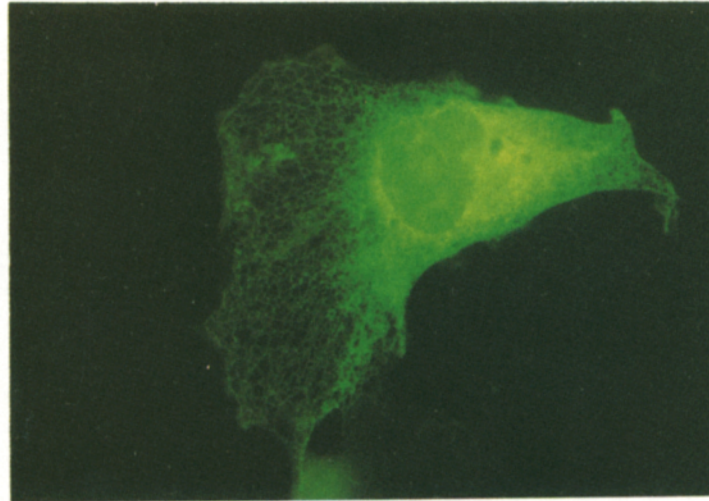
B

CD3 γ

HMT3-2



HMT3-2



WGA

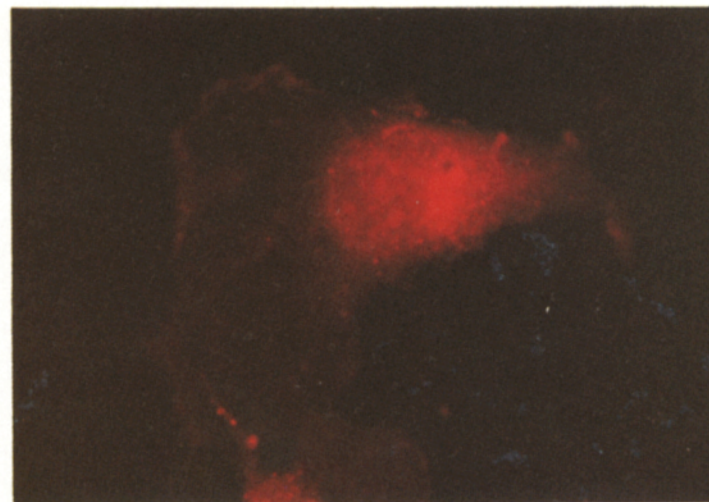


Figure 4.

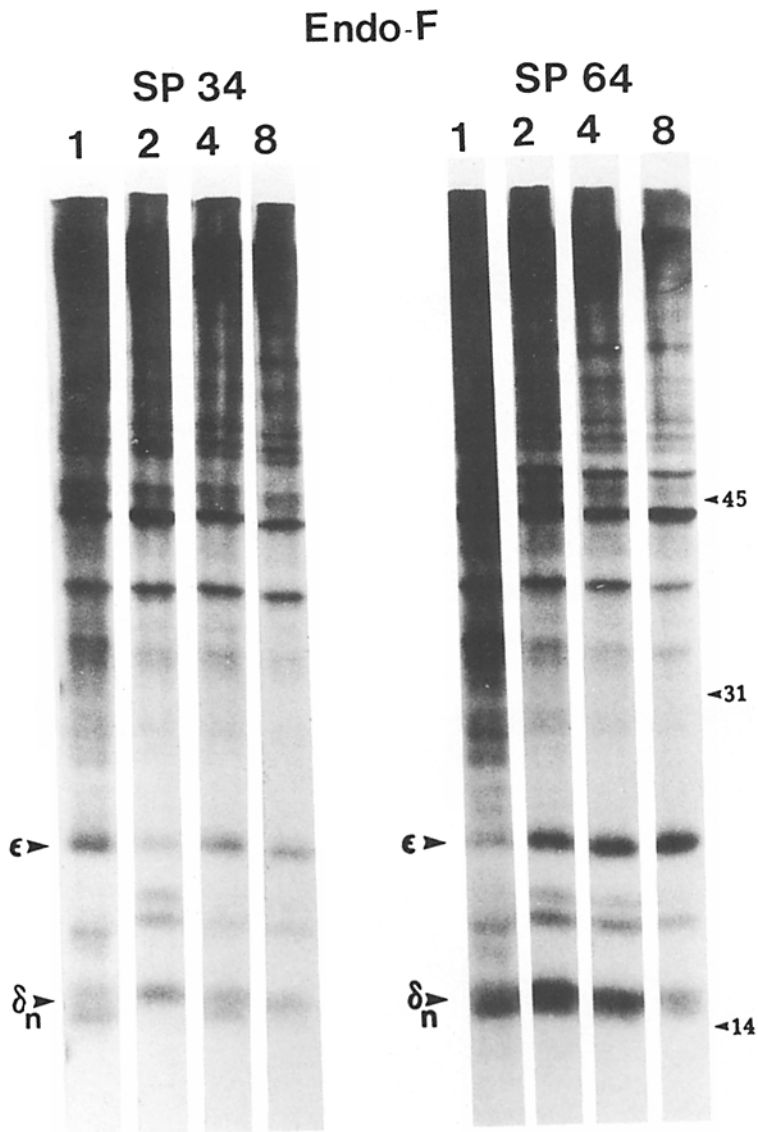


Figure 5. Coexpression of CD3 δ and CD3 ϵ proteins in CHO cells results in subunit association and stabilization of CD3 δ . CHO DUX B11 cells were transfected sequentially with CD3 ϵ and CD3 δ . The cells were pulse-labeled with [35 S]methionine and [35 S]cysteine for 30 min and then chased in complete medium for the indicated times. Labeled cells were lysed in digitonin immunoprecipitation buffer and immunoprecipitated using an antibody specific for CD3 ϵ (SP34, left) or CD3 δ (SP64, right). The washed precipitates were digested with Endo-F and then analyzed by 12.5 SDS/PAGE under reducing conditions. Molecular mass standards (in kilodaltons) are shown. The positions of CD3 ϵ and δ (deglycosylated) are indicated.

prepared from nontransfected CHO cells.) This protein, seen in each lane of *b*, was not glycosylated and was not degraded. The quantity of ϵ precipitated fell little throughout the chase. There was evidence for some degradation of the β chain in these cells since the amount of coprecipitated β chain fell slowly during the chase, nevertheless the β chain, like the δ chain (Fig. 5) was stabilized by association with CD3 ϵ . A comparison of the metabolic half-life of β transfected alone (Fig. 1) and when cotransfected with CD3 ϵ was made using laser scanning densitometry of the autoradiograms (Fig. 9). The half-life of β was raised approximately fourfold from the 1.5 h calculated for β transfected alone, to 6 h in the β/ϵ complex. To determine where in the cell the association of β and CD3 ϵ were taking place immunoprecipitates from each time point were digested with Endo-H (*b*). At all time points the β chain bound to ϵ was sensitive to Endo-H showing that association and stabilization of β took place without transport of either chain to the medial Golgi. The absence of a large excess of TCR β chain (see rationale above for β/ϵ cells) in these cells was confirmed by precipitating cell lysates with an antibody to β (data not shown).

A similar experiment was conducted using the α/β cell cotransfected with the unstable CD3 δ subunit. Fig. 7 *c* shows pulse-chase immunoprecipitation using an antibody to δ (SP64). The antibody precipitated δ and an associated TCR β chain, again there was no visible precipitation of TCR α . The nonspecific protein band was seen again just below β . After an \sim 2-h lag period the intensities of the δ and β chains precipitated during the chase fell rapidly. The experiment demonstrated that although the δ and β chains associated together, they did not stabilize one another. Their half-lives in the cotransfected cells were similar to those observed when they were transfected alone.

Fig. 8 shows analysis of α/β CHO cells that have been cotransfected with two CD3 subunits. *a* shows analysis of such a cell transfected with γ and δ . On the left, the cells have been immunoprecipitated with an antibody that recognizes both γ and δ (SP 64), while on the right, the antibody used (HMT 3.2) was specific for only the γ chain. The left panel showed that the TCR β chain but not TCR α , was precipitated in association with the CD3 subunits. The bands that appeared at 35 kD were the two glycosylated forms of TCR

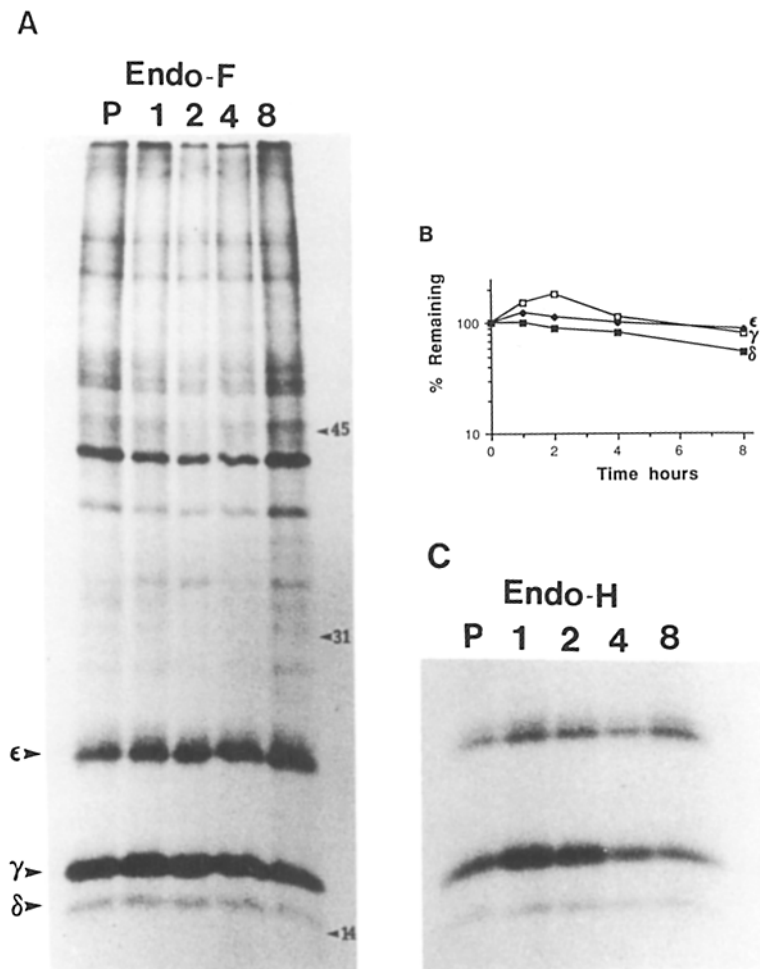


Figure 6. Coexpression of CD3 γ , δ , and ϵ in CHO cells results in assembly of a stable complex. CHO DUX B11 cells transfected with CD3 γ were cotransfected with CD3 δ and CD3 ϵ . The cells were pulse-labeled with [35 S]methionine and [35 S]cysteine for 30 min and then chased in complete medium for the indicated times. Labeled cells were lysed in digitonin immunoprecipitation buffer and immunoprecipitated using an antibody specific for CD3 ϵ (SP34). **A** shows precipitates that were digested with Endo-F before analysis by 12.5 SDS-PAGE under reducing conditions. **B** shows scanning densitometric analysis of the autoradiogram. **C** shows precipitates that were digested with Endo-H. Molecular mass standards (in kilodaltons) are shown on the right. The positions of CD3 γ (deglycosylated), CD3 ϵ and δ (deglycosylated) are indicated.

β , their identity as the β chain was confirmed by Endo-F digestion (see below). Interpretation of the lower bands on the gel was complicated because the antibody used (SP64) recognized both γ and δ chains. The band at 18 kD seen early in the chase was γ with a single *N*-linked oligosaccharide, this was processed during the chase to 25 kD after addition of the second oligosaccharide. Significant levels of the β and γ chains were seen to survive throughout the experiment, whereas the δ chain, seen at 20 kD, was lost during the metabolic chase. At this point it was not possible to determine whether the δ or the γ subunit were stabilizing the cotransfected β chain. The data presented in Fig. 7 *c* showed that δ subunit alone was unable to slow degradation of β and it seemed likely that the β chain was stabilized in these cells by association with the γ subunit. This was confirmed by repeating the experiment using an antibody specific for CD3 γ (*right*). The antibody precipitated the 18- and 25-kD forms of γ and the associated β chain but no δ subunit. The last lane of the right panel shows that the multiple bands seen on the autoradiogram were indeed the β and γ subunits. The immunoprecipitate was digested with Endo-F before electrophoresis after which the glycosylated forms of β migrated at 30 kD and the two forms of γ migrated at 16 kD.

B and **C** show analogous experiments in which the α/β CHO cell was co-transfected with either ϵ and δ or ϵ and γ . In each case the cells were precipitated with an antibody to

ϵ . The β chain but not the α chain precipitated in association with ϵ and the levels of β chain fell slowly during the chase. The presence of coprecipitating γ or δ chains, which were partly obscured in these gels by the ϵ subunit, were confirmed in separate experiments by Endo-F digestion (data not shown). The relative stability of the β chain in each of the cell lines described was determined by laser scanning densitometry and the results are summarized in Fig. 9. It appeared that cotransfection of cells with two CD3 subunits conferred a modest increase in stability on the β chain when compared with transfection with CD3 ϵ alone.

The fate of the α chain in each cell line was determined. To do this CD3/ β complexes were first removed from the lysates by three sequential precipitations with the CD3-specific antibodies. The cleared lysates were then precipitated with an antibody specific for the α chain. The autoradiogram at the base of each panel shows the degradation of the alpha chain in these cells.

Discussion

The biosynthesis of the T cell receptor for antigen provides an intriguing system for a study of the sorting and transport of multiple subunit membrane complexes. Experiments from this laboratory (Alarcon et al., 1988) and others (Koning et al., 1988) have shown that the six subunits of the receptor

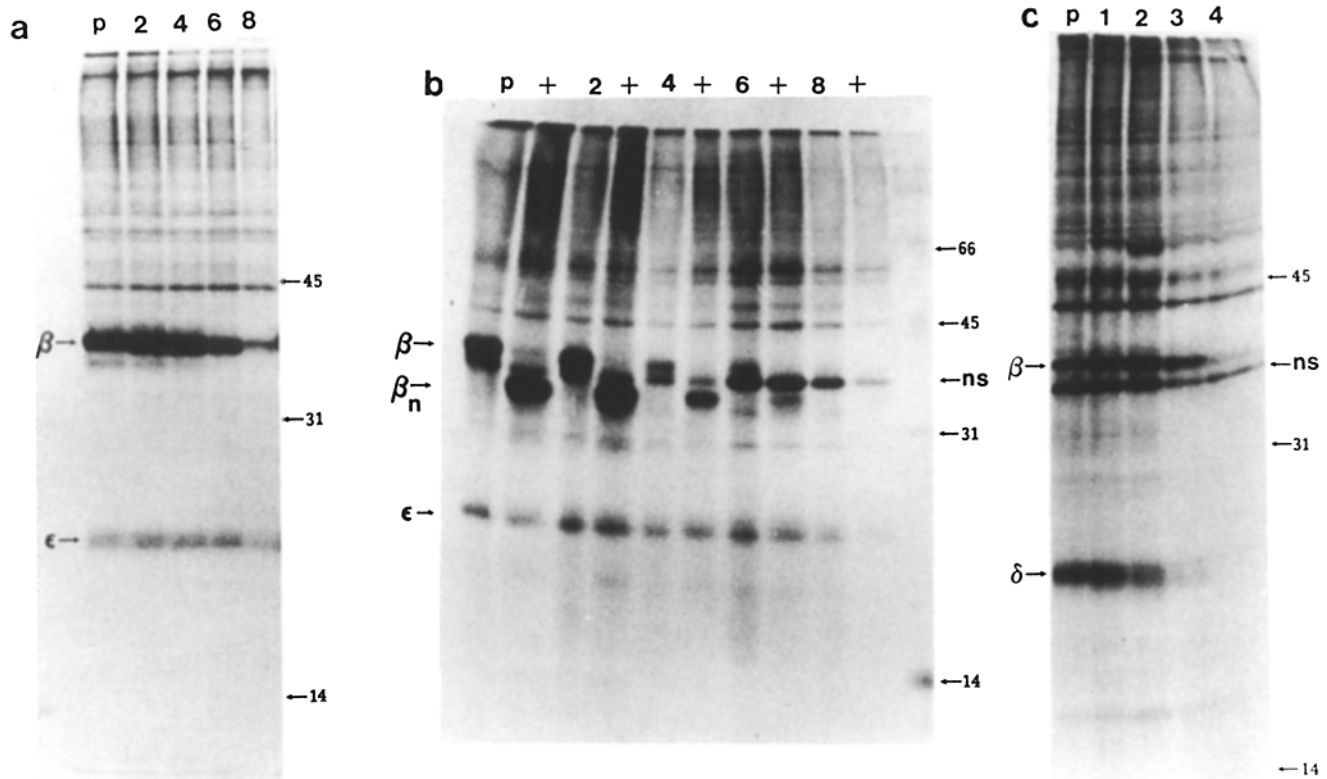


Figure 7. CD3 ϵ but not CD3 δ is able to stabilize TCR β . CHO K cells were cotransfected stably with TCR α and β . Cloned cells were subsequently transfected with either CD3 ϵ or CD3 δ . Each cell line was pulse-labeled with [35 S]methionine and [35 S]cysteine for 30 min and then chased in complete medium for the indicated times. Labeled cells were lysed in digitonin immunoprecipitation buffer and immunoprecipitated using an antibody specific for the transfected CD3 subunit. SP34 was used for the analysis of CD3 ϵ and SP64 used for CD3 δ . *a* shows pulse-chase immunoprecipitation analysis of a CHO cell transfected with $\alpha\beta$ and CD3 ϵ . The positions of ϵ (precipitated by SP 34) and the coprecipitated β chain are indicated. In *b*, half the precipitate for each time point shown in *a* was digested with Endo-H (+). The position of β , deglycosylated β (β_n), and ϵ are indicated. *c* shows analysis of CHO cells transfected with $\alpha\beta$ and CD3 δ . The positions of CD3 δ (precipitated by SP 64) and the coprecipitated β chain are indicated. The nonspecific protein visible in each gel close to the TCR β chain is labeled "ns." All precipitates were analysed by 12.5 SDS-PAGE under reducing conditions. The migration of molecular mass standards (in kilodaltons) is shown.

assemble in the ER shortly after synthesis and that complete assembly is required for movement of the receptor into the Golgi apparatus. Assembly is an ordered process. It has been shown that the CD3 proteins associate with one another first and then bind to individual TCR chains (Alarcon et al., 1988; Koning et al., 1988). It is possible that this interaction facilitates the formation of the disulfide linkage of the α - β heterodimer.

Recent studies suggest that subunits that do not become incorporated into the plasma membrane are degraded intracellularly (Chen et al., 1988; Minami et al., 1987; Sussman et al., 1988; Lippincott-Schwartz et al., 1988). Interestingly, intracellular complexes are susceptible to two different degradation pathways. One pathway is followed by TCR/CD3 complexes that lack only the ζ subunit and takes them through the medial Golgi to secondary lysosomes (Sussman et al., 1988). A second pathway degrades chains before they reach the medial Golgi and is resistant to the effects of lysosomotropic drugs. This second pathway has been referred to as "pre-Golgi degradation" and has been used to account for the intracellular turnover of unassembled TCR α (Lippincott-Schwartz et al., 1988) and CD3 δ chains (Chen et al., 1988).

The proposed degradation of partially assembled receptors is however difficult to reconcile with the presence of stable subcomplexes in the ER of human (Alarcon et al., 1988; Koning et al., 1988) and murine T cell lines (Chen et al., 1988; Bonifacino et al., 1989). To understand more clearly the intracellular processes that regulate the transport of the T cell receptor we have used cDNA transfection to study the fate of its individual components; in particular we have assessed the susceptibility of each chain to rapid intracellular turnover. We have then extended the study to follow the transport of precise combinations of subunits.

Our transfection experiments have identified two pathways that control the transport of the receptor. The first, as predicted from previous studies, was one of rapid intracellular degradation and was followed by TCR α and β chains and CD3 δ . Transport of these glycoproteins within the cell was studied using Endo-H digestion (Kornfeld and Kornfeld, 1985) and the chains were found to be degraded without apparently passing through the medial Golgi (Fig. 3). This was taken as evidence for their degradation in or close to the ER. The second pathway, followed by the CD3 ϵ and γ chains was different. They were metabolically stable and Endo-H analy-

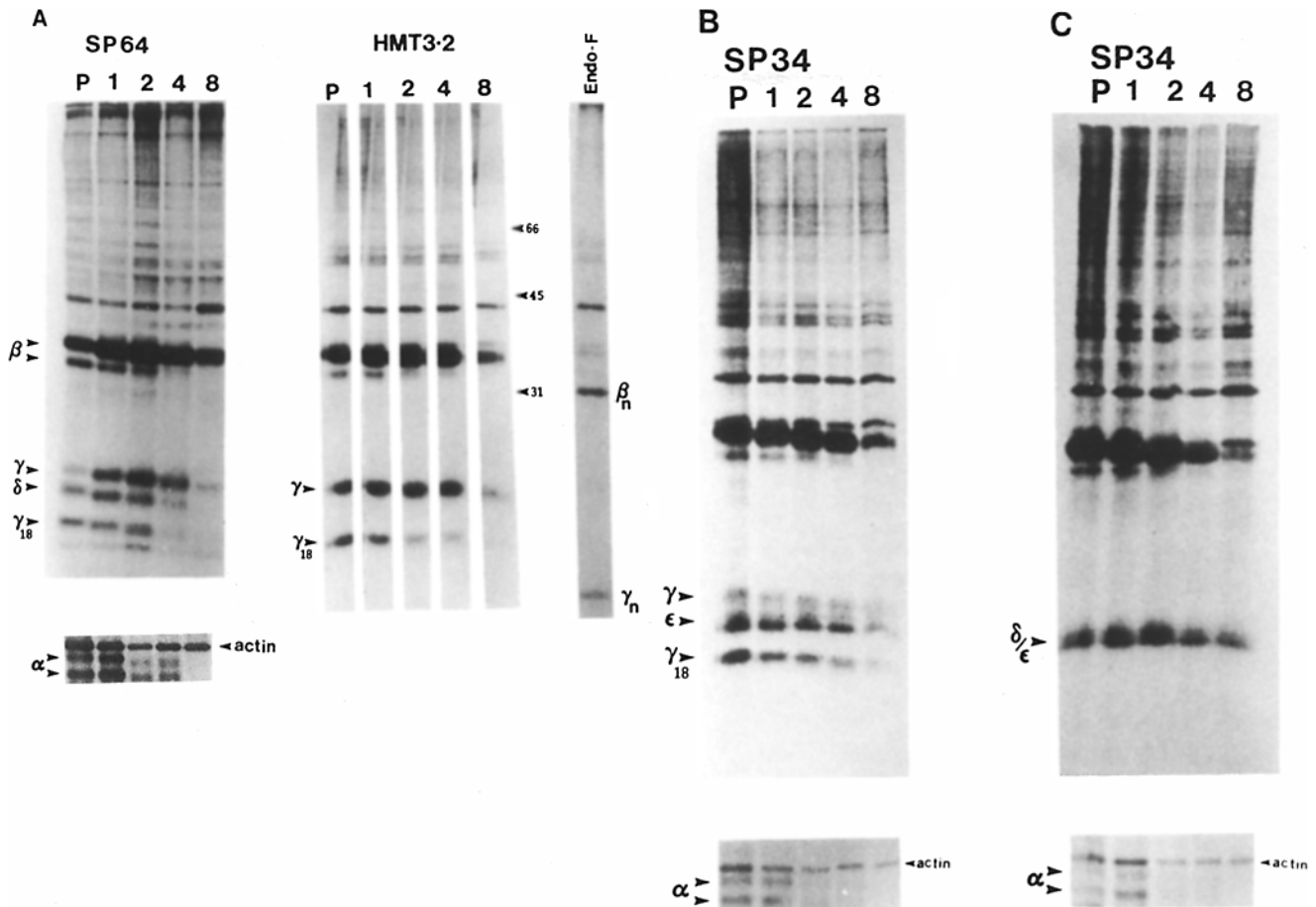


Figure 8. Stabilization of TCR β by two CD3 subunits. CHO K cells were cotransfected stably with TCR α and β . Cloned cells were subsequently transfected with the two CD3 subunits indicated. Each cell line was pulse-labeled with [35 S]methionine and [35 S]cysteine for 30 min and then chased in complete medium for the indicated times. Labeled cells were lysed in digitonin immunoprecipitation buffer and immunoprecipitated using an antibody specific for the transfected CD3 subunits. The lysates were then cleared three times using anti-CD3 antibodies and reprecipitated with an antibody to the α chain (α -F1). Immunoprecipitation of the alpha chain is shown at the base of each panel. (A) The autoradiogram on the left shows pulse-chase immunoprecipitation (SP64 $\gamma\delta$ -specific) analysis of a CHO cell transfected with $\alpha\beta$ and CD3 γ and δ . The positions of γ , δ and the co-precipitated β chain are indicated. The autoradiogram on the right shows analysis of the same cell line carried out using HMT 3.2 (γ -specific). β shows pulse-chase immunoprecipitation (SP34 ϵ -specific) analysis of the $\alpha\beta$ CHO cell co-transfected with CD3 δ and ϵ . C shows analysis of CHO cells transfected with $\alpha\beta$ and CD3 γ and ϵ also precipitated with the ϵ -specific antibody SP34. The positions of CD3 subunits and the coprecipitated β chain are indicated. All precipitates were analyzed by 12.5 SDS-PAGE under reducing conditions. The migration of molecular mass standards (in kilodaltons) is shown.

sis in combination with immunofluorescence experiments on CD3 γ (Fig. 4) suggested that the subunit was retained in the ER. Its retention did not however make it susceptible to degradation.

In a second series of experiments we have used cotransfection to assess the dominance of the two pathways. At the outset there were two possibilities. Either the degraded chains could serve a scavenger function early in the secretory pathway and cause the degradation of chains associated with them, or alternatively as proposed above, the CD3 ϵ and γ chains could stabilize the degraded chains and form a stable core on which the rest of the receptor could assemble. Our results were consistent with a model of receptor stabilization. TCR β was protected from proteolysis through its association with CD3 ϵ or γ and CD3 δ was protected by its association with ϵ . We also showed the formation of a stable $\gamma\delta\epsilon$ complex. This CD3 complex remained sensitive to

Endo-H throughout a prolonged metabolic chase suggesting its retention early in the secretory pathway. This was likely to be the same "pre-Golgi" compartment from which single TCR α and β or CD3 δ were degraded. Interestingly, association and stabilization of the receptor chains showed some subunit specificity, for example, when TCR β and CD3 δ were cotransfected into cells they formed a dimer that was degraded rapidly (Fig. 7 b). It appeared that stability towards proteolysis was not merely a consequence of oligomerization.

cDNA transfection has been used to assess the susceptibility of four ($\alpha\gamma\epsilon\zeta$) components of the murine TCR to degradation (Bonifacino et al., 1989). It is interesting to compare the results from the murine system with those obtained in our study because when taken together the results provide information on the fate of all six chains of the receptor. Bonifacino et al. (1989) showed that the murine ϵ and ζ subunits were

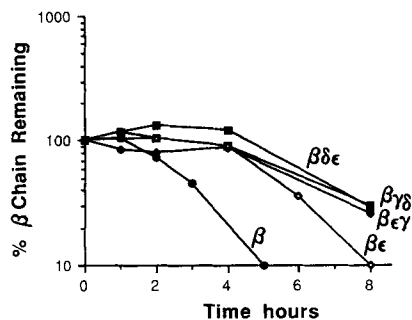


Figure 9. Densitometric analysis of the relative stabilities of different TCR β -CD3 complexes. The autoradiograms shown in Fig. 1 b (β), Fig. 7 a (β - ϵ) and Fig. 8 (β - $\gamma\delta$, β - $\delta\epsilon$, and β - $\gamma\epsilon$) were analyzed using laser densitometry. The relative intensities of the TCR β bands were estimated and plotted as percentage of total TCR β chain precipitated during the pulse label. The actin band was used to normalize data between different lanes and experiments.

stable and that the α and γ chains were degraded. In this respect the murine α and ϵ subunits behaved like their human counterparts. Interestingly, the murine CD3 γ subunit did not. Unlike the murine subunit the human γ chain was not only resistant to pre-Golgi degradation when transfected alone into cells (Fig. 1) but was also able to stabilize a cotransfected β chain (Fig. 8). The human and murine CD3 γ chains are very similar in structure, but a comparison of the deduced amino acid sequences of the two chains reveals that 36 of the 160 amino acids of the mature peptides differ (Krissansen et al., 1987). Because these changes in amino acid composition are concentrated in the extracellular (NH_2 -terminal) domains of the protein that are exposed to the lumen of the ER, they may account for the observed differences in susceptibility to proteolysis. The murine CD3 δ chain has not been expressed alone in cells but since it turns over rapidly in T cells (Chen et al., 1988), it seems probable that it will resemble the human chain (Fig. 1) and be susceptible to pre-Golgi degradation.

Coimmunoprecipitation experiments failed to identify specific associations between CD3 proteins and TCR α . At present the reasons for this are unknown but the data suggest that either the CD3 chains associate preferentially with TCR β , or that the antibodies that we have used in the experiments displace interactions between CD3 proteins and TCR α . The displacement of associated α chains during the immunoprecipitation seems unlikely because the α chain in the cells transfected with two CD3 subunits was degraded. If the chain had associated with either γ or ϵ one might expect it to have survived throughout the metabolic chase. In either case the absence of the α chain from the immunoprecipitates showed that TCR α - β disulfide bonds, which would result in the precipitation of TCR α because it is bound covalently to β , had not been formed during the pulse-labeling or the subsequent chase period. It remains possible that efficient disulfide linkage requires the presence of all the CD3 components and the ζ subunit (Alarcon et al., 1988; Koning et al., 1988). The resistance of the murine ζ chain to proteolysis (Bonifacino et al., 1989) may allow this chain to play an important role in the stabilization of the α chain.

Although we showed the marked stability of complexes containing CD3 ϵ or γ the experiments also provided evidence for an equilibrium between stable and degraded

chains. For example the β chain was lost slowly ($t_{1/2}$ 4 h) from an ϵ/β complex (Figs. 7 a and 9). Because dissociation and subsequent degradation of β took place without the formation of Endo-H-resistant forms it would appear that the equilibrium was established in the ER or certainly without movement of the subunits to the medial Golgi. Interestingly the same equilibrium was not apparent in reconstituted $\gamma\delta\epsilon$ complexes. In this case a significant quantity of the labile δ subunit remained associated with ϵ and γ even after an 8 hour chase. Similar results have been demonstrated for associations between murine CD3 subunits (Bonifacino et al., 1989); for example the murine CD3 ϵ and γ subunits associate to form a dimer that is stable for at least 8 h.

Resolution of the precise mechanisms that allow the degradation of some subunits of the receptor but not others is a complex problem. The unassembled TCR α and β and CD3 δ subunits may be intrinsically sensitive to proteases present in the endoplasmic reticulum. If so, then the stabilization of these chains that occurs as a consequence of association with CD3 γ and ϵ subunits could be mediated by steric or conformational hindrance of recognition by proteases. In this way, exposed protease-sensitive domains would be masked during the assembly of the receptor allowing partial complexes to survive in the ER. Alternatively, it is possible that the labile chains are sorted selectively from the endoplasmic reticulum to an organelle that contains hydrolytic enzymes. Stabilization through associations with CD3 ϵ or γ would result if these subunits were to prevent the sorting of α , β , or δ chains to the proteolytic compartment. The sorting pathway proposes the segregation of destructive proteases away from the bulk flow of the secretory apparatus but the true nature of its endpoint remains obscure. Immunofluorescence labeling of the steady-state distribution of CD3 γ and δ chains (Fig. 4) failed to reveal any intracellular structures that exclude the stable γ chain yet contain the labile δ subunit. Moreover, two lines of evidence preclude the involvement of secondary lysosomes in the hydrolysis of these chains. The degraded chains do not appear to be transported to the distal Golgi (Fig. 3); the site where membrane proteins are sorted for transport to lysosomes (Barriocanal et al., 1986; Green et al., 1987; Geuze et al., 1988) and their catabolism is resistant to the effects of lysosomotropic agents (Chen et al., 1988; Lippincott-Schwartz et al., 1988).

Degradation in or close to the ER does not appear to be restricted to the elements of the T cell receptor. For example, when transfected alone into fibroblasts, the H2 subunit of the human asialoglycoprotein receptor turns over rapidly without processing of its attached oligosaccharides (Shia and Lodish, 1989). Similarly, α -globulin (Stoller and Shields, 1989) and unassembled chains of acetylcholinesterase do not move through the medial Golgi and are degraded by a process that is resistant to lysosomotropic agents (Rotundo et al., 1989). Interestingly, there is some evidence for transport of acetylcholinesterase from the endoplasmic reticulum; unassembled chains appear to pass through the *cis*-Golgi to the sarcoplasmic reticulum of cultured myotubes.

The mechanism of retention of CD3 γ and ϵ is unknown. The most likely possibility is that these chains remain in the ER because they are only partially folded before assembly is complete and as a consequence, their conformation is incompatible with their transport to the Golgi. The importance of oligomerization and the adoption of a correct conforma-

tion for the transport of other multichain proteins to the Golgi has been stressed recently (Rose and Doms, 1988; Kreis and Lodish, 1986; Gething et al., 1986). For example, the membrane glycoprotein of vesicular stomatitis virus and the hemagglutinin spike glycoprotein of influenza virus are retained in the endoplasmic reticulum unless they form correctly folded trimers (Kreis and Lodish, 1986; Gething et al., 1986; Copeland et al., 1986, 1988). Similarly, the heavy chains of immunoglobulin and the major histocompatibility antigens accumulate in the endoplasmic reticulum unless they assemble with their respective light chains (Kvist et al., 1982; Sege et al., 1981; Bole et al., 1986; Hendershot et al., 1987). In many of the cases documented above, retention of partially folded proteins in the endoplasmic reticulum is mediated through associations with BiP (immunoglobulin-binding protein) (Bole et al., 1986; Hendershot et al., 1987). Interestingly, recent studies (Chen et al., 1988; Alarcon et al., 1988) have shown that the chains of the T cell receptor do not bind to immunoglobulin binding protein and suggest that other molecules may retain CD3 γ and ϵ in the ER.

We wish to thank Dr. Rolf Kustner for help in preparing expression vectors containing TCR and CD3 subunits and Ralph Kubo for providing the HMT 3.2 antibody.

T. Wileman is an investigator of the Claudia Adams Barr Program in Cancer Research. These studies were supported in part by National Institutes of Health grants A1-15006 and A1-17651, and American Cancer Society grant no. IM-289E.

Received for publication 26 July 1989 and in revised form 23 November 1989.

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