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The transmembrane anchor of the T-cell antigen receptor β chain contains a structural determinant of pre-Golgi proteolysis

Thomas Wileman,*† Gerald R. Carson,‡ Fei F. Shih,‡ Michael F. Concino,‡§ and Cox Terhorst*
*Laboratory of Molecular Immunology
Dana Farber Cancer Institute
Boston, Massachusetts 02115
‡Protein Expression Group
T-Cell Sciences
Cambridge, Massachusetts 02139-4135

Studies with the T-cell antigen receptor (TCR) have shown that the endoplasmic reticulum, or an organelle closely associated with it, can retain and degrade membrane proteins selectively. The observation that only three (α, β, and δ) of the six ($\alpha\beta\gamma\delta\epsilon\zeta$) subunits of the TCR are susceptible to proteolysis implies that structural features within the labile proteins mark them for degradation. The TCR β chain is degraded in the endoplasmic reticulum, and, in this study, we have started to define the domains of the protein that make it susceptible to proteolysis. The experiments show that the transmembrane anchor and short five-amino-acid cytoplasmic tail of the protein contain a dominant determinant of proteolysis. When these residues were removed from the β chain, the protein became resistant to proteolysis. Even though the resulting ectodomain of the β chain lacked a transmembrane anchor, it was not secreted by cells and was retained in the endoplasmic reticulum. We conclude that retention in the endoplasmic reticulum alone does not lead to degradation. The results suggest that structural features within the membrane anchor of the protein predispose the β chain to proteolysis. This was confirmed by replacing the membrane anchor of the interleukin 2 (IL2) receptor, a protein that was stable within the secretory pathway, with that of the TCR β chain. The unmodified IL2 receptor was transported efficiently to the surface of cells, and an "anchor minus" construct was secreted quantitatively into the culture media. When the membrane anchor of the IL2 receptor was replaced with that of the TCR β chain, the chimera was unable to reach the Golgi apparatus and was degraded rapidly.

† Corresponding author.
§ Present address: Procept, Inc., Cambridge, MA 02139.

Introduction

Many cell-surface receptors and viral glycoproteins are multiple-subunit membrane complexes (Carlin and Merlie, 1986). In many cases the subunits are synthesized at different rates, and the assembly of receptors containing the correct stoichiometry of subunits poses a considerable problem for the cell. Recently it has become clear that the endoplasmic reticulum plays a central role in regulating the transport and the assembly of multiple-subunit complexes (reviewed by Rose and Doms, 1988; Hurtley and Helenius, 1989). Results from many studies have demonstrated that the components of these complexes have to assemble completely before they can leave the endoplasmic reticulum (Bole *et al.,* 1986, Copeland *et al.,* 1986, 1988; Gething *et al.,* 1986, Kreiss and Lodish, 1986). Single subunits and partial complexes are retained in the organelle and brought into contact with proteins and enzymes that catalyze their folding and oligomeric assembly (reviewed Pelham, 1989; Rothman, 1989). Recent studies with the T-cell receptor for antigen (TCR) have shown that the endoplasmic reticulum, or an organelle closely associated with it, also contains proteases. These proteases can degrade subunits that fail to be incorporated into receptors and, in doing so, remove them from the secretory pathway (Lippincott-Schwartz *et al.,* 1988). Proteolysis within the endoplasmic reticulum can demonstrate a high degree of specificity. The TCR is composed of six different membrane proteins ($\alpha\beta\gamma\delta\epsilon\zeta$), but only three of these—TCR α, β, and CD3 δ—are susceptible to proteolysis (Chen *et al.,* 1988, Bonifacino *et al.,* 1989; Wileman *et al.,* 1990a). The others (γ, ϵ, and ζ) are metabolically stable and are retained in the endoplasmic reticulum for prolonged periods. It has been known for some time that an obligate step in the transport of the labile

TCR α and β chains to the plasma membrane of T-cells is their assembly with the CD3 complex (γ, δ, and ϵ) and the ζ homodimer (Ohashi *et al.*, 1985). One reason for this is that assembly of the α and β chains with stable CD3 proteins protects them from degradation in the endoplasmic reticulum and allows them to survive for transport to the Golgi (Bonifacino *et al.*, 1989; Wileman *et al.*, 1990a). Taken together, these studies have shown that an interplay in the endoplasmic reticulum between subunit retention, oligomeric assembly, and selective degradation can determine the composition of receptors that will eventually reach the surface of cells.

These functions of the endoplasmic reticulum are carried out by proteins and enzymes resident in the organelle, and the specificity of their action suggests that there may be some form of molecular recognition. For example, retention may involve recognition of hydrophobic domains that are exposed in partial complexes but are masked during assembly or, at a higher level of resolution, involve recognition of peptide sequences. For example, a C-terminal KDEL sequence present on soluble proteins will lead to their retention in the lumen of the organelle (Munro and Pelham, 1987), and peptide sequences conserved between cytoplasmic domains of some resident membrane proteins have been implicated as retention elements (Nilsson *et al.*, 1989). At present the way in which proteolysis within the endoplasmic reticulum is controlled is unclear. The observed specific degradation of chains of the TCR and other receptors (Shia and Lodish, 1989) suggests that there may be structural elements within these proteins that make them susceptible to proteolysis. Recent studies (Bonifacino *et al.*, 1990) show that the transmembrane anchor of the murine TCR α chain predisposes the chain to degradation. In this study we have taken a similar approach and have started to define the domains of the human TCR β chain that mark the protein for rapid proteolysis soon after synthesis. The experiments show that the transmembrane anchor and short five-amino-acid cytoplasmic tail of the β chain also contain a determinant of proteolysis. When these amino acids are removed from the β chain, the protein survives for prolonged periods in the endoplasmic reticulum. When the membrane anchor of the β chain is fused to an otherwise stable protein, the ectodomain of the interleukin 2 (IL2) receptor, the IL2 receptor becomes a substrate for pre-Golgi proteolysis. The results point to a general role being played by membrane anchors in directing the degradation of susceptible membrane proteins.

Results

Deletion of the transmembrane anchor and cytoplasmic tail prevents rapid degradation of the TCR β chain

The introduction of a premature stop codon (at Leu^{257}) into the c-DNA encoding the TCR β of Jurkat (Figure 1, Table 1) prevented the translation of the transmembrane and cytoplasmic amino acids. c-DNAs encoding the complete β chain and the truncated chain (β-ecto, Figure 1) were transfected stably into Chinese hamster ovary (CHO) cells. Cloned cell lines were pulse-labeled for 30 min and, at the stated time points, were lysed in 1% NP40 and immunoprecipitated with β F1, a monoclonal antibody (Mab) specific for the β chain. Figure 2 shows polyacrylamide gel electrophoresis (PAGE) analysis of the immunoprecipitations. Cells transfected with the complete β chain (left) produced two proteins,

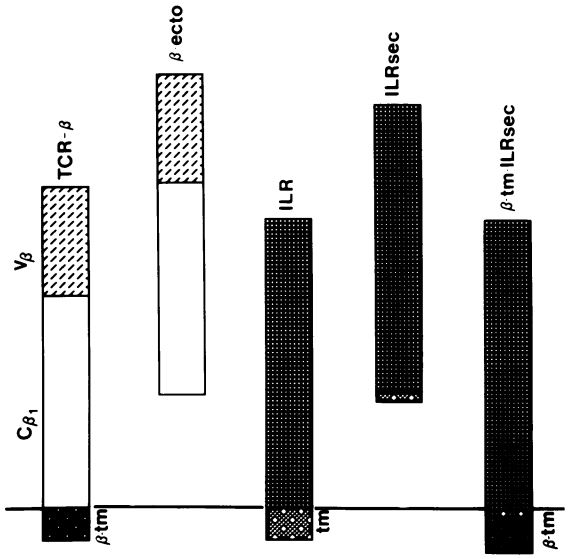
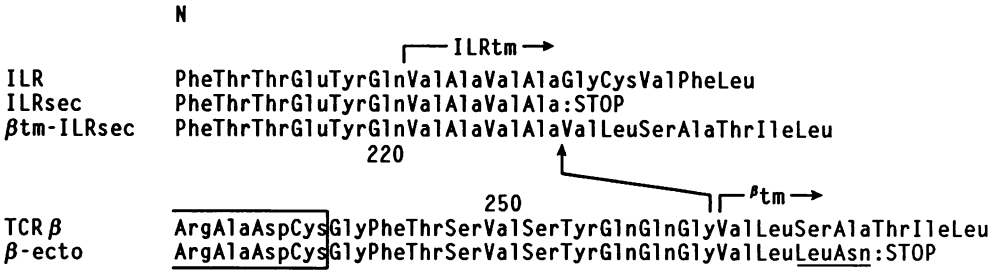


Figure 1. **Schematic representation of parent, truncated, and chimeric receptors.** TCR β. Representation of the TCR β showing relative distribution of amino acids between variable region (Vβ), constant region (Cβ), and membrane anchor (β-tm). β-ecto. The mutant β chain contains the Cβ and Vβ extracellular amino acids, but a stop codon prevents translation of the membrane anchor. ILR. Representation of the unmodified IL2 receptor showing relative distribution amino acids between extracellular domain (ILR) and membrane anchor (tm). ILRsec. The mutant IL2 receptor consists of extracellular amino acids and first four hydrophobic amino acids of the transmembrane domain. A stop codon prevents translation of the rest of the membrane anchor. β-tm ILRsec. The endogenous membrane anchor of the IL2 receptor was replaced by the membrane anchor of the TCR β chain (βtm).

Table 1. Construction of chimeric proteins



The amino acid sequences of the affected areas of the component proteins are shown. ILR. The 15 amino acids around the join between the transmembrane domain at Val#221 and the extracellular residues are shown. ILRsec. The stop codon prevents translation of the transmembrane domain at Ala#224. Four hydrophobic amino acids are retained. βtm-ILRsec. Ala#224 of the IL2 receptor was fused to the first amino acid (Val#256) of the TCR β transmembrane domain. TCR β. The 21 amino acids around the join between the transmembrane domain and the extracellular amino acids of the TCR β chain are shown. The anchor begins at the indicated residue (Val#256). β-ecto. The stop codon prevents translation of the transmembrane domain after the second hydrophobic amino acid Leu#257; the termination primer adds additional Leu and Asp residues. The boxed area indicates the tetrapeptide homologous to the C-terminus of the immunoglobulin λ chain (Gascoigne, 1990).

seen as a major band of 35 kDa and a minor band of 33 kDa. These bands, seen in immunoprecipitates of human T-cells, represented the two glycosylated forms of the β chain, the larger form having two *N*-linked oligosaccharides and the lower band only one.

Figure 2, right, shows a similar analysis of CHO cells transfected with the truncated β chain. Again, two glycosylated forms of the protein were observed. During the chase the smaller form was lost slowly, presumably because of processing to a higher molecular weight on addition of a second oligosaccharide chain. The ectodomain was slightly smaller (32 kDa) than the parent TCR β (35 kDa) chain, the difference corresponding to the lost C-terminal 30 amino acids. The levels of both chains remained constant for the first hour of the chase, but an analysis of the levels of protein remaining at later time points showed that there was a marked difference in stability between the two constructs. The levels of the truncated β chain were constant over the 4 h of the experiment, but the bulk of the full-length β chain was lost from the cells during the third hour of the chase. The experiments were repeated and the chase was extended to 8 h. Figure 3 shows the intracellular stability of the two proteins compared by densitometric analysis of autoradiograms. The graph shows that the ~1 h half-life of the native β chain was increased to 5 h on removal of the transmembrane anchor and short cytoplasmic tail of 5 amino acids.

TCR β chain and ectodomain of receptor fail to reach medial Golgi

To determine how far along the secretory pathway the native and mutant β chains were being transported, we repeated the pulse-chase immunoprecipitations and used endoglycosidase H (Endo-H) to digest precipitations from each time point before electrophoresis. Figure 4A shows an analysis of the transport of the native β chain and shows that the protein was degraded without the appearance of Endo-H resistant forms. Figure 4B shows a similar but extended analysis of the transport of the β chain ectodomain in which culture supernatants were taken and analyzed for the appearance of immunoreactive material. The upper gels show that the ectodomain was lost slowly from cells during an 8-h chase. Even though the truncated β chain lacked a transmembrane anchor, undetectable levels of immunoreactive material could be precipitated from culture supernatants. In separate experiments, overnight culture supernatants taken from confluent monolayers were analyzed with a sensitive TCR β enzyme-linked immunosorbent assay (ELISA). Table 2 shows that the steady-state levels of the transfected β-ecto protein were 10-fold higher than those in cells transfected with the full-length β chain or Jurkat T-cells, yet the levels of protein present in the culture media were undetectable. In the lower gel of Figure 4B, immunoprecipitates from each time point were digested with

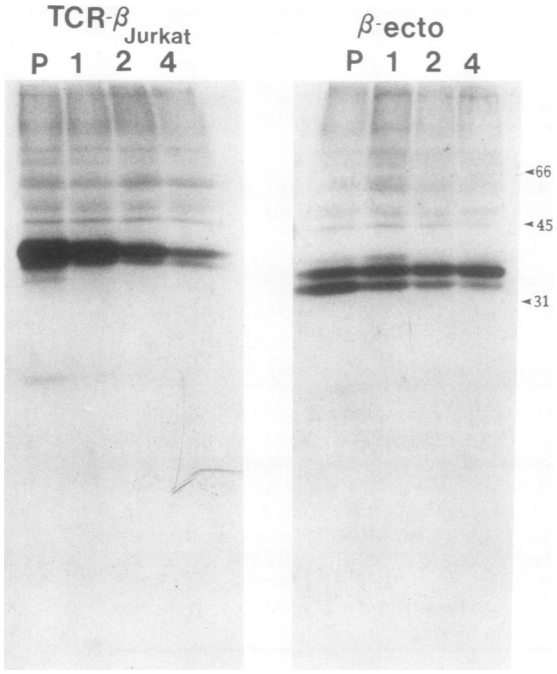


Figure 2. **Relative stability of parent and truncated TCR β chain.** CHO cells were transfected stably with either the full-length chain (TCR-β) or the anchor minus mutant (β-ecto). Cells were pulse-labeled with [^{35}S]-methionine/cysteine for 30 min at 37°C and then chased for the indicated hourly time intervals. After lysis in 1% NP-40, the transfected proteins were immunoprecipitated with the TCR β chain–specific Mab β-F1 and analyzed by 12.5% SDS-PAGE. The positions of molecular mass markers (kilodaltons) are shown.

Endo-H; the figure shows that a small quantity of the lower protein band became resistant to the enzyme but that the bulk of the ectodomain of the β chain remained sensitive to Endo-H during the 8-h chase. Taken together, the results showed that the native β chain was degraded without passing through the medial Golgi, whereas the ectodomain of the TCR β chain was stable and was retained for the most part in or close to the endoplasmic reticulum.

Ectodomain of 55-kDa α chain of IL2 receptor becomes susceptible to proteolysis when fused to membrane anchor of TCR β chain

The observation that the TCR β chain could be rendered stable after removal of its transmembrane and cytosolic amino acids suggested that these residues may contain an element that marked the β chain for proteolysis. To test this possibility, we fused these same amino acids to a reporter protein that was otherwise stable within the secretory pathway. The ectodomain of the IL2 receptor was chosen for these studies.

The 55-kDa chain of the human IL2 receptor (Figure 1) consists of an *N*-terminal extracellular domain of 219 amino acids joined to 19 hydrophobic amino acids that form a transmembrane anchor. An additional 13 amino acids form a cytoplasmic tail (Treiger *et al.*, 1986). Figure 5A shows pulse/chase immunoprecipitation analysis of fibroblasts transfected stably with a c-DNA encoding a full-length IL2 receptor. As predicted, the antibody precipitated a protein of 43 kDa from pulse-labeled cells and during the chase the molecular mass of the receptor increased to a broad band of ~55 kDa. The molecular weight increase was attributed to a posttranslational addition of charged residues to O-linked sugars as they pass through the Golgi apparatus. The IL2 receptor contains two *N*-linked oligosaccharides, and the gel shows that during the chase the quantity of Endo-H sensitive material decreased, again indicating movement of the protein into the Golgi.

A premature stop codon was introduced at Ala^{224} of the molecule to prevent translation of the transmembrane amino acids and the cytoplasmic tail. The construct (ILRsec Figure 1, Table 1) was transfected stably into CHO cells, and its intracellular transport was analyzed by

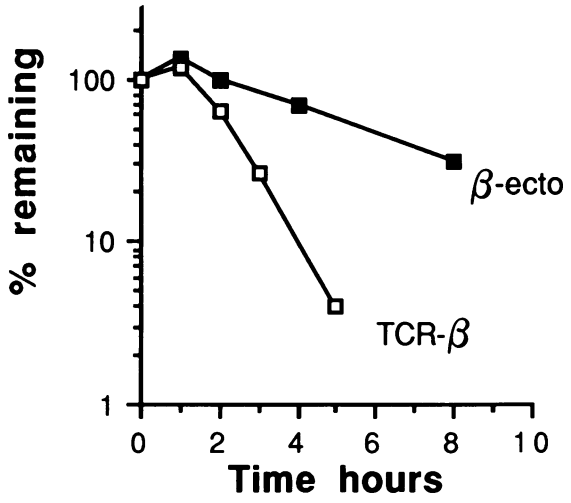


Figure 3. **Comparison of intracellular survival of parent and truncated TCR β chain determined by scanning densitometry.** Pulse-chase immunoprecipitations were carried out as described in the legend to Figure 2, but the metabolic chase was extended to 8 h. The levels of precipitated protein at each time point were determined by densitometric scanning of autoradiograms. The quantity of β chain present was expressed as a percentage of the protein precipitated from pulse-labeled cells. The data points represent the average of three estimations.

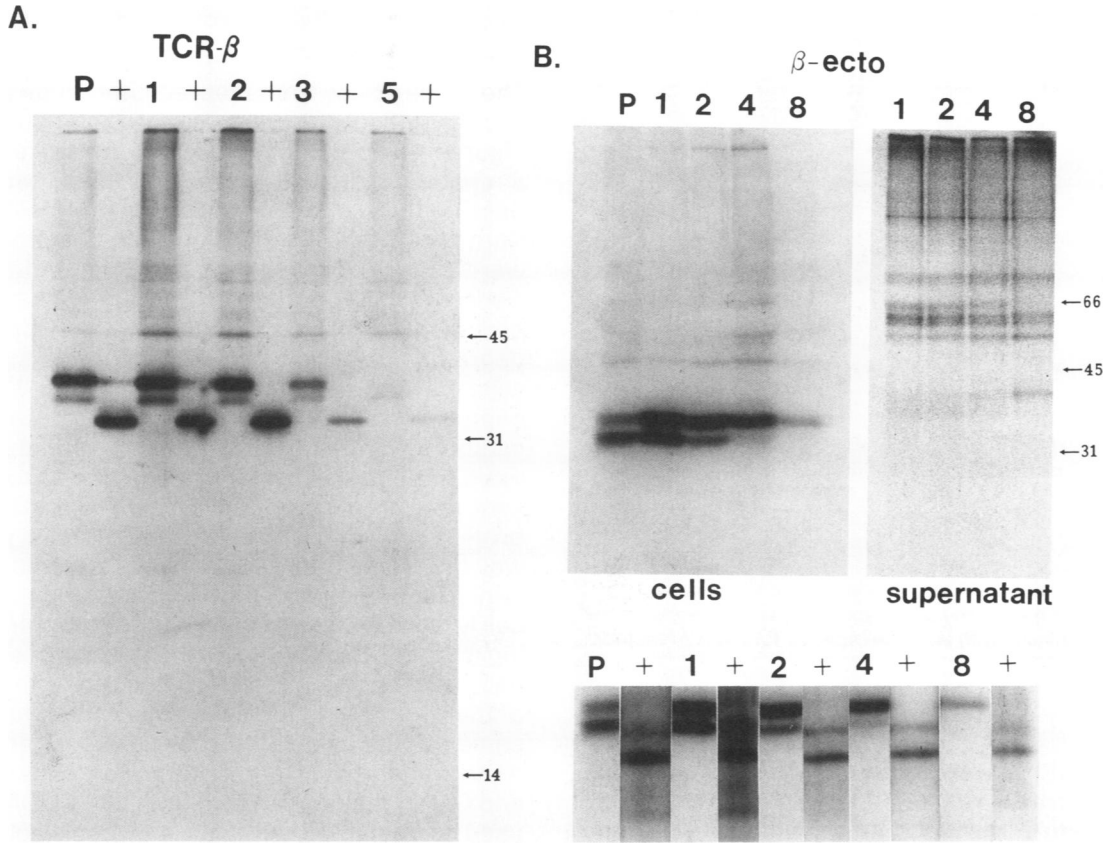


Figure 4. **The parent and truncated TCR β chain do not reach the medial Golgi.** The transfected cell lines were analyzed by pulse-chase immunoprecipitation and SDS-PAGE as described in the legend to Figure 2. (A) Unmodified TCR-β chain. At each time point, one-half the precipitated protein was digested with Endo-H (+). (B) Anchor minus, mutant β-ecto. The top gels compare immunoprecipitations of cell lysates (left) and culture supernatants (right). In the lower panel, one-half the protein precipitated from the cell lysates was digested with Endo-H (+). Molecular mass markers (kilodaltons) are shown.

pulse-chase immunoprecipitation. Figure 5B shows the secretion of the protein into the culture supernatant. The antibody precipitated a protein of 40 kDa from pulse-labeled cells, and the quantity of immunoprecipitable material remaining in the cell lysates fell with time. During this period there was a quantitative appearance of labeled protein in the cell media. Significantly, the gel shows that the ectodomain of the receptor was not susceptible to proteolysis, because ~80% of the pulse-labeled material could be recovered within 4 h from culture supernatants. Two-thirds of this material was resistant to Endo-H, indicating its secretion via the Golgi apparatus.

Having established that the IL2 receptor was not susceptible to proteolysis within the secretory pathway, we next replaced the transmembrane and cytosolic amino acids of the receptor with those of the TCR β chain. CHO cells were transfected stably with the βtm-ILRsec construct (Figure 1, Table 1) and analyzed by the pulse/chase immunoprecipitation protocol described above. The experiment was performed to see whether the transport of the fusion protein would be governed by the IL2 receptor moiety and be transported intact from the endoplasmic reticulum to the cell surface, or whether the fused amino acids derived from the TCR β chain would cause rapid intracellular proteolysis of the molecule. Immunoprecipitation of cell lysates with a Mab specific for the IL2 receptor (7G7) (Figure 6A) precipitated a protein with a molecular mass of 40 kDa. During the chase the quantity of immunoprecipitable material within the cell decreased with time. The intracellular half-life of 45 min, calculated by scanning densitometry (Figure 6B), showed that the fusion protein was degraded slightly faster than the parent β chain ($T_{1/2}$ 60 min). The experiment was repeated with a second Mab (AM 92.3) specific for the IL2 receptor, and identical results were recorded (data not shown). At each time point, one-half of each sample was di-

Table 2. Relative levels and distribution of transfected proteins

Transfected protein	TCR-β ELISA (OD units) Cells (5×10^6)	Supernatant (total in 8 ml)
Jurkat (T-cells)	20	<0.05
TCR-β	35	<0.05
β-ecto	200	<0.05
	IL2R ELISA (ng)	
βtm-ILRsec	0.9	<0.1
ILRsec	1.8	13.6

Transfected CHO cells were grown as confluent monolayers in 10-cm^2 dishes. Cells were lifted from the dishes using EDTA, counted and lysed in 1% NP40, and assayed for TCR β chain using a two-antibody ELISA. The table shows the levels of β chain in cell lysates and in membrane-filtered (0.45 μm) overnight culture supernatants (adjusted to represent the total in 8 ml). Jurkat T-cells were used as an internal standard. CHO cells expressing IL2R epitopes were assayed similarly with an ELISA specific for the IL2 receptor.

gested with Endo-H before electrophoresis. The fusion protein remained sensitive to digestion with Endo-H throughout the chase period, suggesting that, like the full-length β chain, the protein was degraded before it could reach the medial Golgi.

Degradation of fusion proteins does not take place in lysosomes

The pulse-chase immunoprecipitation analysis in conjunction with Endo-H digestion shown in Figures 4 and 6 suggested that the labile TCR β chain constructs were degraded without passing through the medial Golgi (Kornfeld and Kornfeld, 1985). To confirm that the proteins were being degraded in a pre-Golgi and not a lysosomal compartment, the effects of a series of inhibitors of lysosome function on protein degradation were tested. To perform this analysis, we employed the TCR β chain and IL2 receptor–specific ELISAs to follow intracellular catabolism (Wileman *et al.*, 1990b). In brief, cells were treated with cycloheximide at 4°C to prevent new protein synthesis, and the remaining protein was chased into the degradative pathway by warming to 37°C in nutrient media containing cycloheximide. At suitable time points, cell lysates were taken and assayed for remaining antigen. Table 3 shows that the intracellular half-life of each construct, calculated by the use of the cycloheximide chase and ELISA, was the same as that calculated previously by densitometric analysis of autoradiograms (Figures 3 and 6B). In the next experiments, the ELISA was used to monitor degradation in each cell line incubated with 100 μM chloroquine, 50 mM ammonium chloride, or 100 μg/ml leupeptin. The data presented in Table 3 showed that incu-

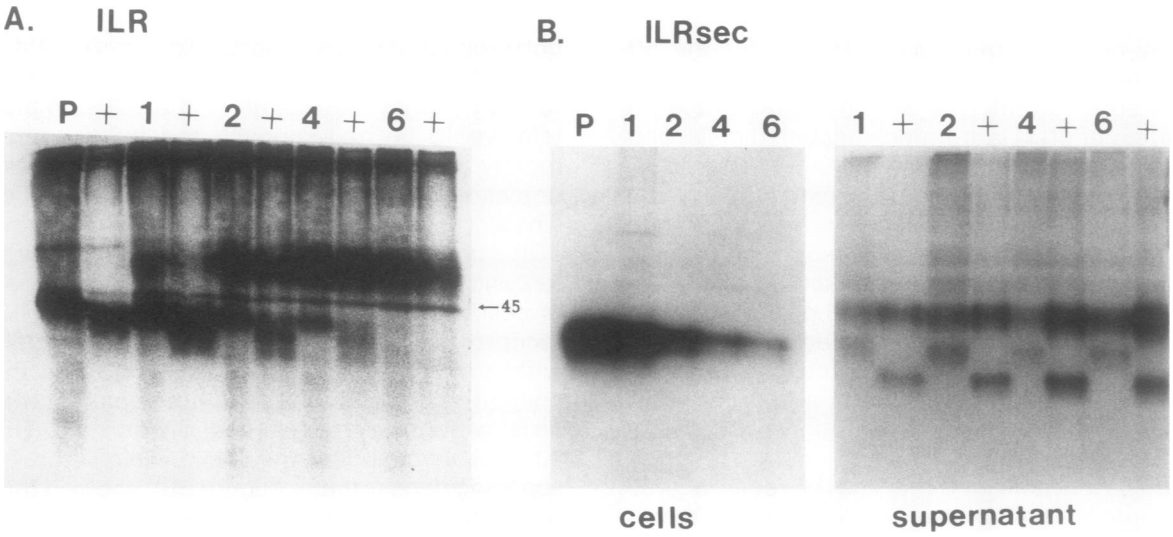


Figure 5. **Intracellular processing of unmodified and truncated IL2 receptor.** Transfected cells were analyzed by pulse-chase immunoprecipitation and SDS-PAGE analysis as described in the legend to Figure 2. (A) The IL2 receptor was precipitated from cell lysates with Mab 7G7. At each time point half the precipitated protein was digested with Endo-H (+). (B) The "anchor minus" IL2 receptor was precipitated from cell lysates (left) and culture supernatants (right) using the Mab 7G7. At each time point half the protein precipitated from culture supernatants was digested with Endo-H (+).

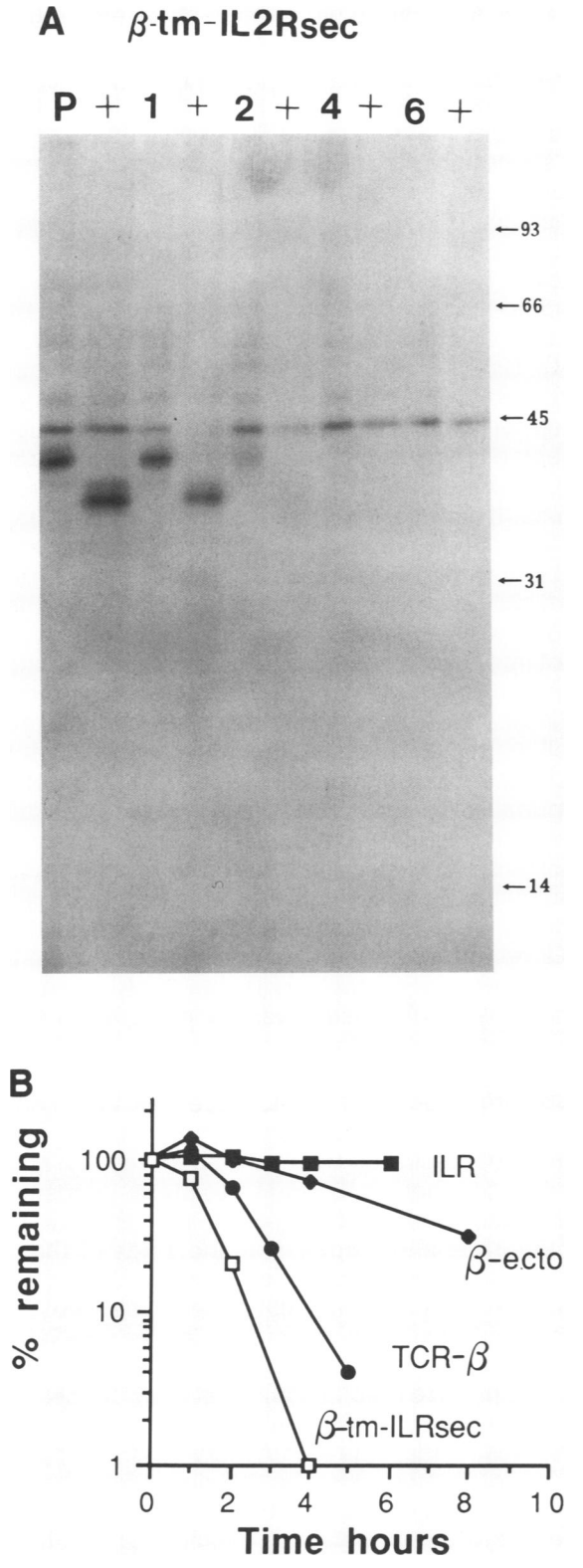


Figure 6. **The chimeric receptor βtm-ILRsec is degraded without reaching the medial Golgi.** The cytosolic and transmembrane amino acids of the IL2 receptor were replaced by those of the TCR β chain. (A) CHO cells transfected stably with the resulting βtm-ILRsec chimera were analyzed by pulse-chase immunoprecipitation and SDS-PAGE as described in the legend to Figure 2. Cell lysates were precipitated with a Mab against the IL2 receptor (7G7). At each time point, one-half the protein precipitated was digested with Endo-H (+). Molecular mass markers (kilodaltons) are shown. (B) The relative levels of the βtm-ILRsec chimera remaining at each time point were estimated by scanning densitometry of autoradiograms (see legend to Figure 3). They are compared with a similar analysis of the IL2 receptor (ILR), unmodified TCR β chain (TCR-β), and anchor minus TCR β chain (β-ecto).

bation of cells with these agents had little effect on the intracellular survival of the constructs, even though these same concentrations of drugs severely reduce the activity of secondary lysosomes (Seglen, 1983). These experiments confirmed that the membrane anchor of the β chain was causing the βtm-ILRsec chimera to be degraded in or close to the endoplasmic reticulum, rather than in lysosomes.

Degradation rates of fusion proteins do not change when transport between endoplasmic reticulum and Golgi apparatus is blocked

Monesin, carbonyl cyanide m-chlorophenylhydrazone (CCCP), and brefeldin A slow the movement of proteins from the endoplasmic reticulum to the Golgi apparatus (Tartakoff 1983, 1986; Fujiwara *et al.,* 1988, Lippincott-Schwartz *et al.,* 1989). In the following experiment, these drugs were used to see whether the relative stability of the transfected proteins would change when their movement from the endoplasmic reticulum was disrupted. The experiments were designed to resolve the issue of whether retention in the endoplasmic reticulum alone would predispose proteins to rapid proteolysis. Control experiments showed that, when cells transfected with the IL2 receptor were incubated with monensin (10 μM) or CCCP (30 μM), the receptor failed to acquire Endo-H–resistant oligosaccharides during the metabolic chase (data not shown). These results indicated that the drugs blocked the movement of the IL2 into the medial Golgi. Next, CHO cells transfected with the different constructs were incubated with the ionophores as described above, or with brefeldin-A, and the cycloheximide chase protocol was used to monitor their effects on the stability of the transfected proteins. Table 3 shows that the relative stability of the native proteins (TCR β, IL2 receptor), their respective ectodomains (β-ecto, ILRsec), or the βtm-ILRsec chimera did not change when transport from the endoplasmic reticulum was slowed. The

Table 3. Effects of drugs on degradation of fusion proteins

	Half-life (min)			
	TCRβ	βecto	ILR	βtmILRsec
Control	50	305	>10 h	41
NH_4Cl (50 mM)	58	320		45
Chloroquine (100 μM)	61	285		40
Leupeptin (100 μg/ml)	50			48
CCCP (30 μM)	65	265	>10 h	45
Monensin (10 μM)	60	330	>10 h	40
Brefeldin A (10 μg/ml)	54	293	>10 h	40

The rate of degradation of the transfected mutant and fusion proteins was determined with the chain-specific ELISAs and a cycloheximide chase. Cells were preincubated with the indicated concentrations of drugs for 1 h before the addition of cycloheximide and the drug was present during the chase. The half-life of each chain was estimated from linear portions of degradation curves. The degradation half-lives, calculated under control conditions with the cycloheximide chase assay, are the same as those calculated by densitometric analysis of autoradiograms obtained from pulse-chase immunoprecipitations (see Figure 6).

data suggested that the susceptibility of the chains to proteolysis was independent of their rate of transport from the endoplasmic reticulum.

Discussion

Plasma membrane proteins are transported by a constitutive flow of membrane and fluid from the endoplasmic reticulum through the Golgi stacks to the cell surface (reviewed by Pfeffer and Rothman 1987). Recent studies on the T-cell antigen receptor have shown that the six individual chains of the receptor ($\alpha\beta\gamma\delta\epsilon\zeta$) have to assemble together correctly if any of them are to follow this route to the plasma membrane. Single subunits and partial receptor complexes are unable to reach the plasma membrane and are arrested in transport before they reach the Golgi apparatus (Alarcon *et al.*, 1988; Chen *et al.*, 1988; Lippincott-Schwartz *et al.*, 1988). These sequestered chains show different sensitivities to a proteolytic activity that is associated with the endoplasmic reticulum (Chen *et al.*, 1988; Lippincott-Schwartz *et al.*, 1988). The α, β, and δ subunits are susceptible to proteolysis and are degraded within 2–3 h of synthesis, whereas the ϵ, γ, and ζ chains are resistant to proteolysis and have prolonged metabolic half-lives (Bonifacino *et al.*, 1989; Wileman *et al.*, 1990a,b). These results demonstrate that the endoplasmic reticulum, or an organelle closely associated with it, can show a high degree of selectivity in the proteins that it degrades.

In this study we have started to define the structural features of the TCR β chain that make it susceptible to proteolysis in the endoplasmic reticulum. The extracellular domain of the TCR β chain consists (depending on the precise sequence of the Vβ region) of some 280 amino acids. These are held in the membrane by a 27-amino-acid transmembrane domain followed by a short 5-amino-acid cytoplasmic tail (Yanagi *et al.*, 1984). Deletion of the membrane anchor from the TCR β chain resulted in its loss of susceptibility to proteolysis (Figure 3) and suggested that these residues were important structural determinants in the proteolytic process. If this were so, then it seemed likely that these residues would be able to confer susceptibility to degradation on another protein. Preliminary experiments (Figure 5) showed that the 55-kDa chain of the human IL2 receptor was not intrinsically susceptible to degradation in the endoplasmic reticulum and would be a good reporter protein for such an experiment. Both the full-length and the "anchor-minus" mutant ILRsec were transported quantitatively from the endoplasmic reticulum to the Golgi apparatus and then to the plasma membrane. Most important, when transport from the endoplasmic reticulum to the Golgi was blocked by pharmacological intervention, the ectodomain of the IL2 receptor was stable and did not become a substrate for proteolysis (Table 3). A chimeric molecule (βtm-ILRsec) was constructed to see whether the transmembrane anchor of the β chain would cause degradation of the extracellular amino acids of the IL2 receptor. When analyzed by pulse-chase immunoprecipitation, the chimeric receptor was degraded rapidly and failed to acquire Endo-H–resistant oligosaccharides. The results implied that the transmembrane and cytosolic amino acids of the β chain did indeed contain an element that could be transferred to an otherwise stable protein and mark these fused amino acids for pre-Golgi degradation.

The precise mechanisms that allow for selective degradation of proteins before they reach the Golgi apparatus are unknown. For example, it is not known whether the degraded chains are intrinsically susceptible to proteases resident in the endoplasmic reticulum or whether the chains are in some way recognized specifically by accessory molecules and marked for degradation. This could involve their priming for degradation in the endoplasmic reticulum itself

or their recognition for sorting and transport to an organelle containing proteases. The experiments described here have demonstrated the importance of the β chain membrane anchor in making proteins fused to it susceptible to degradation. The same conclusions have been drawn from recent studies concentrating on the fate of the murine TCR α chain. The transmembrane 23 amino acids of the α chain also mark luminal amino acids fused to it for rapid proteolysis (Bonifacino *et al.*, 1990).

It appears from these studies that the involvement of transmembrane anchors as determinants of proteolysis may be a general phenomenon. If this is the case, then it would be interesting to know whether there is any indication that predisposition to proteolysis results from a recognition element, such as specific amino acid sequence, that is conserved between transmembrane domains. Table 4 shows the sequences of the transmembrane domains of membrane proteins that have so far been shown to be susceptible to degradation soon after synthesis. The examples include the H2 subunit of the asialoglycoprotein receptor (Amara *et al.*, 1989; Shia and Lodish, 1989), the CD3 γ and δ subunits of the murine TCR (Chen *et al.*, 1988; Bonifacino *et al.*, 1989), and the CD3 δ subunit of the human TCR (Wileman *et al.*, 1990b). Shown for comparison in the same table are the membrane anchors of proteins that are homologous to their listed counterparts, yet are resistant to pre-Golgi degradation. These are the H1 subunit of the asialoglycoprotein receptor (Spiess and Lodish, 1985), human and murine CD3 ε (Gold *et al.*, 1986), and human CD3 γ (Krissansen *et al.*, 1986, 1987). The table shows that there are no obvious amino acid sequences that are conserved between the chains that are susceptible to degradation but absent from their stable counterparts. A minimum degradation/assembly element has been mapped to eight amino acids, L-R^+-I-L-L-L-K^+-V, that lie near the center of the membrane anchor of the TCR α chain (Manolios *et al.*, 1990). Although this precise sequence is absent from the other chains listed, a motif consisting of a lysine residue separated by four nonpolar amino acids from a charged residue (Y-E^--I-L-L-G-K^+-A) is present in the transmembrane domain of the human TCR β chain. Whether this sequence alone directs the degradation of the β chain, or the IL2 receptor chimera, must await further deletion analysis.

It is worth noting that the involvement of membrane anchors in directing the degradation

Table 4. Amino acid sequences of transmembrane domains

		T1/2 (hours)
	C N	
ASOR H1	L-L-L-L-S-L-G-L-S-L-L-L-L-V-V-V-C-V-I-G	>10.0
ASOR H2	F-S-L-L-A-L-S-F-N-I-L-L-L-V-V-I-C-V-T-G	0.5
	N (-) C	
CD3 γ mouse	I-G-T-I-S-G-F-I-F-A-Ⓔ-V-I-S-I-F-F-L-A-L-G-V-Y-L-I-A-G	1.0
CD3 γ human	A-A-T-I-S-G-F-L-F-A-Ⓔ-I-V-S-I-F-V-L-A-V-G-V-Y-F-I-A-G	6.0
CD3 δ mouse	S-G-T-M-A-G-V-I-F-I-Ⓓ-L-I-A-T-L-L-L-A-L-G-V-Y-C-F-A-G	1.0
CD3 δ human	P-A-T-V-A-G-I-I-V-T-Ⓓ-V-I-A-T-L-L-L-A-L-G-V-F-C-F-A-G	1.0
CD3 ε mouse	L-T-A-V-A-I-I-I-I-V-Ⓓ-I-C-I-T-L-G-L-L-M-V-I-Y-Y-W-S-K	>8.0
CD3 ε human	V-M-S-V-A-T-I-V-I-V-Ⓓ-I-C-I-T-G-G-L-L-L-L-V-Y-Y-W-S-K	>8.0
	(-)	
	(-) (+)	
TCR β human	I-L-Y-Ⓔ-I-L-L-G-Ⓚ-A-T-L-Y-A-V-L-V-S-A-L-V-L-M-A-M-V	0.9
TCR α mouse	N-F-Q-N-L-S-V-M-G-L-Ⓡ-I-L-L-L-Ⓚ-V-A-G-F-N-L-L-M-T-L	1.0
	(+) (+)	
	cytosol→	

The table shows the amino acid sequences of the membrane anchors of proteins that are susceptible to proteolysis and compares them with the sequences of homologous proteins that are resistant to degradation. The half-lives of the proteins shown in the table are published values that have been obtained from studies that have followed the degradation of each protein expressed alone in cells. References are given in the text. The table lists the two subunits (H1 and H2) of the asialoglycoprotein receptor (ASOR), the three (γ, δ, and ε) members of the murine and human CD3 complex, and the TCR α and β chains. The asialoglycoprotein is a type-2 membrane protein, and its C-terminus is exposed to the lumen of the endoplasmic reticulum. Charged residues are outline letters. The minimum degradation/assembly sequence motif of the TCR α chain (Manolios *et al.*, 1990) is underlined.

of proteins other than TCR α and β subunits has not yet been established. Furthermore, proteins that lack hydrophobic transmembrane domains can also become susceptible to pre-Golgi proteolysis. For example, when fused to the heterologous signal peptide of β-lactamase, α-globin, which is normally resident in the cytosol, translocates into the endoplasmic reticulum and is degraded rapidly by a nonlysosomal process (Stoller and Shields, 1988). Mutant forms (PiZ and Pi $Null_{Hong\ Kong}$) of human α_1-antitrypsin that fail to enter the Golgi apparatus of hepatocytes turn over rapidly (Le *et al.,* 1990), as do incompletely assembled chains of apolipoprotein B-100 (Sato *et al.,* 1990). Given these observations, it seems possible that the transmembrane domains of the TCR α and β chains could exert their effects through a mechanism that does not involve the recognition of amino acid sequences and that they may function in a less specific way. For example, association with the limiting membrane of the endoplasmic reticulum may be their important function. In this respect it is interesting that 50% of the apolipoprotein B-100 present in the endoplasmic reticulum, where it is for the most part degraded, is bound to the membrane of the organelle (Bamberger and Lane, 1988; Bostrom *et al.,* 1988). The examples of degradation of anchor minus proteins listed above (α-globin, α_1-antitrypsin, and apolipoprotein B-100) imply that the proteases responsible for degradation are active in the lumen of the secretory pathway rather than in the cytosol. It is possible that the membrane anchors studied thus far exert their destabilizing effect by sequestering attached proteins to domains of the endoplasmic reticulum that are rich in these proteases. Alternatively, they may impose conformational restraints on attached proteins that expose protease-sensitive sites.

It is significant that retention in the endoplasmic reticulum alone does not lead to proteolysis. Experiments show that the TCR α (Bonifacino *et al.,* 1990) and β ectodomains (Figure 4 and Table 2) have a long residence time in the endoplasmic reticulum, yet they are relatively stable. Moreover, when transport from the endoplasmic reticulum is slowed by treating cells with ionophores or brefeldin-A (TCR β, Table 3; TCR α, Bonifacino *et al.,* 1990), there is no increased predisposition of these luminal residues to degradation. It appears unlikely, therefore, that the TCR α and β ectodomains evade proteolysis simply because, without membrane anchors, they are more mobile in the secretory pathway. A recent study (Gascoigne, 1990) shows that the ectodomain of the murine TCR β chain can be secreted by cells if further deletions into the constant region are made. Removal of 10 amino acids from the C-terminal of the $C\beta_1$ domain leaves a C-terminal tetrapeptide (R-A-D-C, see boxed amino acids 241–245 in Table 1) that is homologous to the C-terminal of immunoglobulin λ chain. When transfected into B-cells or T-cells, the truncated murine TCR β chain is stable, but, unlike the anchor minus β-ecto construct described in this study, the shorter ectodomain is no longer retained in the endoplasmic reticulum but is secreted efficiently. These observations imply either that the 10 deleted C-terminal amino acids constitute a retention element within the β chain that is recognized by the luminal contents of the endoplasmic reticulum or that the newly exposed C-terminal tetrapeptide contains information that allows the efficient movement of the chain into the secretory pathway of lymphocytes. The fate of the shorter β chain ectodomain in fibroblasts has not been tested.

In summary, the relative stabilities of the native and modified proteins studied here show that the membrane anchor of the TCR β chain, like the TCR α chain, contains an important determinant of pre-Golgi proteolysis. It is possible that a structural motif (±-I-L-L-X-K) is all that is required to direct the degradation of these two proteins. The absence of this motif from other membrane proteins that are degraded in the endoplasmic reticulum, however, argues against its obligate recognition by the proteolytic apparatus of the organelle. Interestingly, because retention in the endoplasmic reticulum alone is unable to render proteins susceptible to proteolysis, it is possible, as an alternative mechanism, that the TCR α and β membrane anchors exert their effects through an interaction with domains of the endoplasmic reticulum that regulate access to proteases. In T-cells, the TCR β chain is stabilized in the endoplasmic reticulum when it assembles with the ϵ and γ chains of the CD3 complex (Wileman *et al.,* 1990a). It is likely that the CD3 proteins protect the β chain by masking the determinants of proteolysis that reside in its membrane anchor.

Materials and methods

Cells

CHO cell lines K1 were obtained from ATCC (Rockville, MD). CHO cells were maintained in 5% CO_2 at 37°C in a base medium of RPMI supplemented with glutamine (2 mM); 10 μg/ml each of adenosine, thymidine, and deoxyadenosine; and 15 μg/ml proline. Mouse L-cells expressing the full-length α chain of the IL2 receptor were a generous gift from

Dr. Warner Greene (Duke University, Durham, NC). Both lines were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine and buffered with 10% CO_2. All media contained 8% heat-inactivated fetal calf serum (FCS). Tissue culture media were purchased from GIBCO (Grand Island, NY) or Sigma (St. Louis, MO).

Construction of truncated and fusion proteins

Standard molecular biology protocols (Maniatis *et al.*, 1982) were used throughout. The structures of the fusion proteins are shown as diagrams in Figure 1, and the amino acid sequences around the fusions are shown in Table 1. The plasmid pYT35 (Yanagi *et al.*, 1984) provided the cDNA encoding the TCR β chain derived from the T-cell line Jurkat. The plasmid pIL2R3 (Leonard *et al.*, 1984) provided a cDNA encoding the full-length (55 kDa) α chain of the IL2 receptor. β-ecto: The cDNA encoding the TCR β chain of Jurkat was opened at the Fok 1 cleavage site at nucleotide #867; the four-base-pair overhang was filled with Klenow and ligated to the universal translation terminator (Pharmacia, Piscataway, NJ). This resulted in addition of codons for two new amino acids (Leu and Asn) C-terminal to Leu #257 and a stop codon (Table 1). ILRsec: The ILRsec anchor minus construct has been described previously (Treiger *et al.*, 1986). In brief, a translation termination codon was introduced at the *Nae* I restriction site (base pair #912) of the cDNA. This caused translation of the protein to stop at Ala#224, the fourth hydrophobic amino acid of the transmembrane anchor. βtm-ILRsec: The cDNA encoding the TCR β chain of Jurkat was subcloned into pUC19 and opened using a Ppu M1 digestion (base pair #868). The resulting three-base-pair overhang was end-filled without loss of reading frame. The 5′ untranslated region and the remaining nucleotides encoding the extracellular domain of the β chain were removed using *Eco*RI. In separate experiments, the nucleotides encoding the entire extracellular domain of the IL2 receptor were removed from pIL2R3 using a *Nae* I/*Eco*RI restriction digest. The *Nae* I site left an in-frame blunt end, allowing the fragment to be subcloned into the end-filled Ppu M1/*Eco*RI-cut plasmid containing the β chain transmembrane domain.

```
        Nae 1 ↓                          Ppu M1
5′  GTAGCAGTGGCC                    GTCCTGTCTGCCACC
3′  CATCGTCACCGG                      GACAGACGGTGG

          GTAGCAGTGGCCGTCCTGTCTGCCACC
          ValAlaValAlaValLeuSerAlaThr
            ← ILRsec |├──────βtm →
```

Transfection

The proteins were expressed in CHO cells by subcloning the relevant reading frames into a mammalian expression vector containing a transcriptional unit constructed from the adenovirus major late promoter linked in tandem to the resistance gene, xanthine-guanine phosphoribosyltransferase (*gpt*) (Mulligan and Berg, 1981) driven by the SV 40 early promoter. CHO K1 cells were transfected using the calcium phosphate precipitation procedure (Graham and Van der Eb, 1973) and selected in growth media supplemented with hypoxanthine (135 μg/ml), xanthine (13 μg/ml), and mycophenolic acid (10 μg/ml).

Antibodies

W76 and IdentiTy β-F1 were provided by T-Cell Sciences (Cambridge, MA) and are specific for TCR β. 7G7 (Rubin *et al.*, 1985) and AM 92.3 (T-Cell Sciences) are specific for the human IL2 receptor.

Metabolic labeling, immunoprecipitation, and electrophoresis

Transfected cells were labeled metabolically with [^{35}S]-methionine and [^{35}S]-cysteine using Trans ^{35}S-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 ^{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 and immunoprecipitated as described previously (Alarcon *et al.*, 1988). Immunoprecipitates were washed extensively and solubilized in reducing sample buffer in preparation for sodium dodecyl sulfate (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, we reprecipitated lysates with the relevant antibodies. In each case <10% of the original protein remained in the lysate.

Endoglycosidase treatment

Endo-H was purchased from Genzyme (Boston, MA); 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, then diluted to 50 μl with 50 mM phosphate buffer, pH 6.0, containing 50 mU of enzyme.

Laser scanning densitometry

Quantitation of protein bands was performed by scanning autoradiograms with a laser densitometer (LKB Ultroscan XL). A Gaussian integration method was used to estimate the intensity of bands (Gelscan XL software package, LKB, Piscataway, NJ). The actin band was used to normalize data between different lanes and different experiments.

ELISA for TCR β and IL2 receptor

The capture antibody, W76 for TCR β, was adsorbed onto Immulon 96-well plates (Dynatec Laboratories, Chantilly, VA) as a 2-μg/ml solution in phosphate-buffered saline (PBS). Plates were washed with PBS and blocked for 2 h with PBS containing 0.05% Tween 20 and 10% bovine serum albumin (BSA). Antigen samples, diluted to 200 μl with blocking buffer, were applied to drained wells and incubated for 2 h at 37°C. Bound TCR β chain was visualized in washed wells with horseradish peroxidase coupled to β F1, diluted in PBS containing 30% FCS, 6% BSA, and 0.75% NP40. The Cell-free IL2R test kit (T-Cell Sciences) was used to estimate IL2 receptor epitopes in cell supernatants and lysates. Bound peroxidase was determined colorimetrically by addition of *o*-phenylenediamine and H_2O_2.

Cycloheximide-chase ELISA

Transfected cells were grown to 90% confluency in 10-cm^2 dishes. Cells were fed for 1 h with fresh growth media and then preincubated at 4°C for 30 min with 10 μg/ml cycloheximide in RPMI. To start the chase, cells were warmed to 37°C in RPMI containing 10 μg/ml cycloheximide. At suitable time points, the media was removed and monolayers were lysed in 300 μl immunoprecipitation buffer. Lysates were left on ice for 1 h, insoluble material was pelleted by centrifugation, and supernatants (100-μl aliquots) were as-

sayed for antigen with the relevant ELISA. Drugs were added for 1 h at 37°C before preincubation with cycloheximide and then kept in the incubation media throughout the experiment. The preincubation steps were omitted from the experiments using CCCP; instead, cells were preincubated with glucose-free RPMI and then chased with the same media containing cycloheximide and 30 μM CCCP. Details of this assay have been published previously (Wileman *et al.*, 1990b).

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