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Degradation of T-cell receptor chains in the endoplasmic reticulum is inhibited by inhibitors of cysteine proteases

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The endoplasmic reticulum, or an organelle closely associated with it, contains proteases that can be used to remove partially assembled or improperly folded proteins. Very little is known at present about the types of protease that degrade these proteins. The β chain and cluster of differentiation (CD)3 δ subunit of the human T-cell antigen receptor (TCR) are degraded shortly after synthesis. In this study Chinese hamster ovary (CHO) cells transfected with either β or δ were incubated with a panel of protease inhibitors, and the rates of degradation of the transfected proteins were followed using chain-specific enzyme-linked immunosorbent assays (ELISAs). Of the protease inhibitors tested, degradation of both chains was highly sensitive to sulfhydryl reagents and peptidyl inhibitors of cysteine proteases. Concentrations of inhibitors that produced near complete inhibition of degradation in the endoplasmic reticulum did not cause gross changes in cellular ATP levels nor did they significantly slow constitutive secretion from CHO cells. The inhibitors did not affect the ability of CHO cells to synthesize and assemble disulphide-linked TCR ζ dimers. We conclude that the protease inhibitors were not toxic to cells and did not affect the biosynthetic activity of the endoplasmic reticulum. Furthermore, they did not alter the ability of the endoplasmic reticulum to deliver its content to the Golgi apparatus. Taken together, these results suggest that the cysteine protease inhibitors slow degradation in the endoplasmic reticulum through an action on cysteine proteases. The results imply that the endoplasmic reticulum contains cysteine proteases that can be used to remove retained proteins.

Introduction

One function of the endoplasmic reticulum is to deliver membrane proteins, and proteins that are to be secreted by cells, to the Golgi apparatus. Many studies have shown that proteins must fold correctly before they are delivered into the secretory pathway, and proteins that fail to attain a correct tertiary conformation after synthesis, or multiple subunit complexes that do not assemble completely, are retained by the organelle (Copeland *et al.,* 1986, 1988; reviewed by Rose and Doms, 1988; Hurtley and Helenius, 1989; Pelham, 1989). This process offers a convenient means of controlling the structure of proteins that enter the secretory pathway, but it poses a problem for the cell because proteins that are unable to enter the Golgi apparatus will accumulate in the endoplasmic reticulum. It appears that the endoplasmic reticulum uses two methods to minimize this accumulation of protein. First, resident proteins of the lumen of the organelle, e.g., grp78/BiP and protein disulphide isomerase, bind to many newly synthesized proteins and possibly catalyze their folding (Bole *et al.,* 1986; Bullied and Freedman, 1988; reviewed by Rothman, 1989). Second, the endoplasmic reticulum, or an organelle closely associated with it, contains proteases that can be used to remove pools of retained proteins (Lippincott-Schwartz *et al.* 1988, reviewed by Klausner and Sitia 1990).

Good examples of this balance between synthesis, retention, and degradation have been provided from studies on the biosynthesis and transport of the T-cell antigen receptor (TCR).[1]

[1] Abbreviations used: CD, cluster of differentiation; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IL2, interleukin 2; NSF, N-ethylmaleimide sensitive factor; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCR, T-cell antigen receptor; TLCK, tosly-lysine chloromethyl ketone; TPCK, tosyl-phenylalanine chloromethyl ketone; ZPCK, carbobenzoxy phenylalanine chloromethyl ketone.

The receptor is a multimeric complex made from six different transmembrane proteins (TCR α and β, cluster of differentiation [CD]3 γ, δ, ϵ, and ζ); however, only seven-member complexes with a minimum stoichiometry of ($\alpha\beta\gamma\delta\epsilon\zeta$-$\zeta$) (Carson *et al.*, 1991; Hall *et al.*, 1991) or eight-member complexes ($\alpha\beta\gamma\delta\epsilon\epsilon\zeta$-$\zeta$) (Blumberg *et al.*, 1990; reviewed by Exley *et al.*, 1991) are transported efficiently to the cell surface. Single subunits of the receptor and partial receptor complexes can assemble together in the endoplasmic reticulum but they are not transported to the Golgi apparatus. These retained components of the receptor are degraded in the endoplasmic reticulum at a rate that is governed by their subunit composition. The α, β, and CD3 δ subunits are particularly sensitive to proteolysis and are degraded within 1–3 h of synthesis, whereas the γ, ϵ, and ζ subunits, or receptor subcomplexes that contain them, are more stable and have longer half-lives (Chen *et al.*, 1988; Lippincott-Schwartz *et al.*, 1988; Bonifacino *et al.*, 1989; Wileman *et al.*, 1990a). Degradation in the endoplasmic reticulum is not restricted to the proteins of the TCR. Proteases have been shown to degrade many other partially assembled receptors (Amara *et al.*, 1989; Rotundo *et al.*, 1989; Shia and Lodish, 1989; Blount *et al.*, 1990; Sato *et al.*, 1990; Sitia *et al.*, 1990) and misfolded proteins (Stoller and Shields, 1989; Cheng *et al.*, 1990; Koppelman and Cresswell, 1990; Le *et al.*, 1990).

A major focus of recent studies has been to define the subcellular site of proteolysis. Experiments that have followed the maturation of N-linked oligosaccharides attached to susceptible proteins suggest that proteins are broken down before they reach the cis or medial Golgi. In many cases degradation is resistant to drugs that inhibit lysosome function and is unaffected by conditions that block vesicular transport between the endoplasmic reticulum and the Golgi apparatus (Lippincott-Schwartz *et al.*, 1988; Amara *et al.*, 1989; Bonifacino *et al.*, 1989; Le *et al.*, 1990; Wileman *et al.*, 1990b). Agents that deplete the endoplasmic reticulum of calcium ions also block the movement of proteins from the endoplasmic reticulum (Lodish and Kong, 1989), yet these conditions accelerate degradation of newly synthesized T-cell receptor chains (Wileman *et al.*, 1991). All these experiments suggest that proteolysis occurs very early in the secretory pathway, possibly in the endoplasmic reticulum itself.

Although the subcellular site of "pre-Golgi" proteolysis has received much attention, little is known about the nature of these potentially novel proteases. In this present study we have analyzed the ability of protease inhibitors to inhibit degradation. The β chain and CD3 δ subunit of the human T-cell receptor represent two structurally different substrates for proteolysis in the endoplasmic reticulum, and we have used Chinese hamster ovary (CHO) cells transfected with these proteins to characterize protease activity. The effects of a panel of protease inhibitors on degradation have been studied and the results show that degradation of TCR β and CD3 δ is highly sensitive to sulfhydryl reagents and peptide inhibitors of cysteine proteases. The results suggest that cysteine proteases play a role in the removal of excess newly synthesized proteins from the endoplasmic reticulum.

Results

Degradation of TCR β and CD3 δ can be followed rapidly using an enzyme-linked immunosorbent assay (ELISA)

Degradation of TCR β and CD3 δ in transfected CHO cells can be analyzed using a chain-specific ELISA, and we have used this protocol as a rapid and efficient monitor of compounds that might inhibit proteolysis. In this assay (Wileman *et al.*, 1990b) cycloheximide is used to block new protein synthesis, and chains that have already been synthesized are chased into the degradative pathway by warming cells to 37°C. Cell lysates taken at increasing time intervals are assayed for remaining antigen using chain-specific ELISAs. Figure 1 compares two ways of following the loss of immunoreactive material from cells and illustrates the use of the ELISA assay. In panel A, CHO cells transfected with either TCR β or CD3 δ were pulse labeled with ^{35}S-methionine for 30 min and then chased in complete growth media for increasing time intervals. At the indicated times, cells were lysed in 1% NP 40 and immunoprecipitated using the chain-specific monoclonal antibodies βF1 (TCR β) and SP 64 (CD3 δ). When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the levels of labeled protein immunoprecipitated from cell lysates during the chase are seen to fall, and densitometric analysis (Wileman *et al.*, 1990a,b) of autoradiographs has shown that, after an approximate 1-h lag period, TCR β and CD3 δ are lost from cells with a half-life of 1 h. Panel B shows the loss of immunoreactive material from cells monitored using β- and δ-specific ELISAs during a cycloheximide chase. Again, β and δ are lost from cell

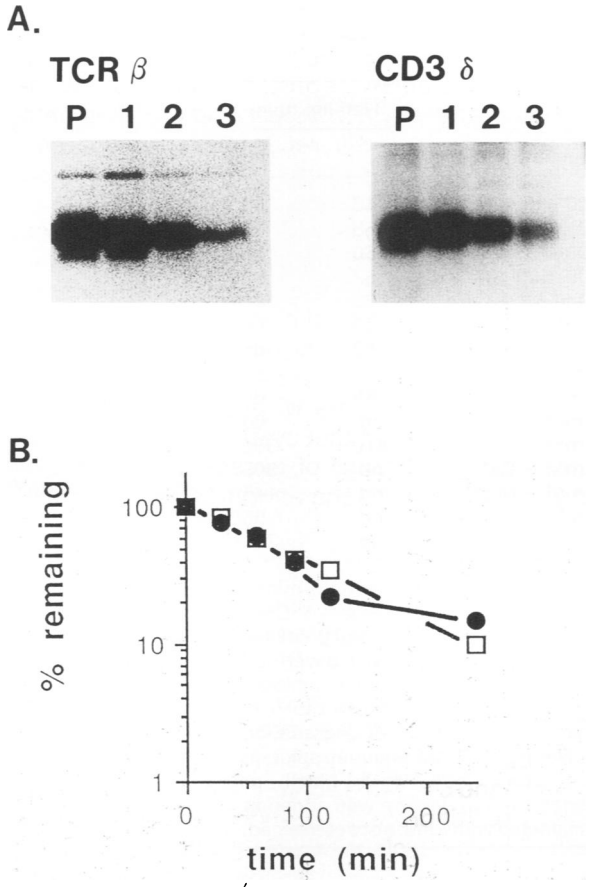

Figure 1. **Degradation of subunits followed using pulse-chase immunoprecipitation or a cycloheximide chase and a subunit-specific ELISA.** (Panel A) Pulse-chase immunoprecipitation. CHO cells transfected with TCR β or CD3 δ were pulse-labeled with ^{35}S-methionine/cysteine for 30 min at 37°C and then chased in complete medium for the indicated hourly time intervals. After lysis in 1% NP 40 the transfected proteins were immunoprecipitated with subunit-specific antibodies (β-F1 for TCR β and SP 64 for CD3 δ) and analyzed by 12.5% SDS-PAGE under reducing conditions. (Panel B) Cycloheximide-chase. Transfected cells were warmed to 37°C in culture media containing 10 μg/ml cycloheximide. At the indicated time points, cell aliquots (10^6 cells) were lysed in 1% NP 40 and the quantity of transfected chain remaining was determined using a subunit-specific immunoassay β (●) δ (□).

lysates with half-lives of ~1 h. The speed of the cycloheximide chase/ELISA lends itself to the rapid calculation of degradation rates, and this method was used in subsequent experiments to screen the effects of a panel of protease inhibitors on degradation.

Cysteine proteases inhibit protein degradation in the endoplasmic reticulum

Aspartyl, serine, cysteine, and metal proteases comprise the four mechanistic classes of proteases that are accepted at present by the International Union of Biochemistry. In the first experiment, the effects of recognized inhibitors of aspartyl and serine proteases on the degradation of TCR β and CD3 δ were tested, and the results are shown in Table 1. Essentially Table 1 shows that the degradation of TCR β or CD3 δ was not affected by these classes of protease inhibitor. The experiment included phenylmethylsulfonyl fluoride (PMSF) and 3,4 dichloroisocoumarin, two general inhibitors of serine proteases, and also chymostatin and tosly-lysine chloromethyl ketone (TLCK), inhibitors of chymotrypsin and trypsin-like serine proteases, respectively. Pepstatin, the one inhibitor of aspartyl proteases tested, was also without effect.

The next class of compounds tested were the inhibitors of metal proteases. Many metalloproteases hydrolyze the peptide bond on the amino side of hydrophobic amino acids, and carbobenzoxy dipeptides containing such amino acids, for example Z-gly-phe or Z-gly-ala, can inhibit such enzymes. Metal proteases also require divalent cations and can therefore be inhibited by metal chelators. The effects of 1,10-phenanthroline, a metal chelator, and a panel of dipeptidyl metal protease inhibitors were tested. Table 1 shows that the dipeptidyl substrates were unable to slow degradation of β or δ. Interestingly, 1,10-phenanthroline slowed degradation of both chains, and an analysis of dose response curves (not shown) suggested that maximum effects were produced between 0.5 and 1 mM. These concentrations of 1,10-phenanthroline extended the half-life of each chain from 1 h (rate 0.018 min^{-1}) to over 6 h (rate 0.002 min^{-1}). 1,10-Phenanthroline is not a competitive substrate of metal-dependent proteases but inhibits metal-dependent proteases through its ability to chelate divalent metal ions. To see if an ability to chelate metal ions was a determinant of the action of 1,10-phenanthroline on degradation, the inhibitor was preincubated with equimolar concentrations of different divalent cations before its addition to cells. Phenanthroline binds weakly to Ca^{++} yet binds more strongly to other divalent cations. The results at the bottom of Table 1 show that preincubation of the drug with Ca^{++} failed to inhibit its activity but preincubation of 1,10-phenanthroline with Mg^{++}, or to a greater extent Mn^{++}, reversed its effects on degradation. Interestingly, Zn^{++}, for which 1,10-phenanthroline has a high affinity, was unable to reverse its effects.

The last group of inhibitors to be analyzed were inhibitors of the thiol-dependent or cys-

Table 1. Effect of inhibitors of serine, aspartyl, and metal proteases on degradation of TCR β and CD3 δ

Inhibitor	Class	Concentration	Half-life (min) β	Half-life (min) δ
PMSF	Serine	200 μM	56	62
3,4,diisocoumarin	Serine	50 μM	58	64
Chymostatin	Serine	100 μM	60	65
TLCK	Serine	100–500 μM	62	69
Pepstatin	Aspartyl	10 μM	65	68
CBZ isoleu-phe	Metal	1 mM	58	60
CBZ gly-phe	Metal	1 mM	63	66
CBZ gly-ala	Metal	1 mM	56	55
CBZ gly-leu	Metal	3 mM	65	60
CBZ ala-phe	Metal	3 mM	nd	60
Phenanthroline	Metal	1 mM	410	380
Phenanthroline		1 mM + Ca^{++}	nd	365
Phenanthroline		1 mM + Mg^{++}	nd	145
Phenanthroline		1 mM + Mn^{++}	nd	55
Phenanthroline		1 mM + Zn^{++}	nd	376

The degradation of TCR β and CD3 δ in transfected CHO cells was determined using the cycloheximide chase assay described in the legend to Figure 1. Cells were preincubated for 2 h at 37°C with the indicated concentration of protease inhibitor in HEPES-buffered RPMI, pH 7.4. Cells were then washed and warmed to 37°C in HEPES-buffered RPMI containing the same concentration of inhibitor and cycloheximide. Preincubation with phenanthroline was carried out for 1 h. The half-life of each chain was calculated from linear portions of degradation curves. Phenanthroline was prechelated by diluting a 10 mM aqueous solution with an equal volume of 10 mM solutions of either $CaCl_2$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$. After incubation at room temperature for 1 h, the mixture was added at a final concentration of 1 mM to CHO cells transfected with δ. nd, not determined.

teine proteases. Cysteine proteases are particularly sensitive to sulphydryl reagents, and the effects of N-ethylmaleimide and iodoacetamide on degradation were tested. Figure 2 shows dose-response curves for the effects of these agents. Low concentrations (5–10 μM) of both reagents caused a near complete inhibition of degradation of δ and β. At maximum effect, these reagents raised the half-life of both chains to over 4 h (rate 0.004 min^{-1}). Certain chloromethylketones can act as relatively specific inhibitors of cysteine proteases (reviewed by Shaw, 1990), and the effects of three peptidyl chloromethylketones on degradation were tested. Degradation of β and δ was inhibited in a dose-dependent manner by tosyl-phenylalanine chloromethyl ketone (TPCK), carbobenzoxy phenylalanine chloromethyl ketone (ZPCK) (Figure 2), and 10 μM penylbutyryl-leu-norleu-chloromethyl ketone (data not shown). A structural analogue, TLCK, which reacts preferentially with trypsin-like serine proteases, was ineffective over the same concentration range. Calpains are calcium-dependent cysteine proteases (Murachi, 1983; Mellgren, 1987) and inhibitors of calpains also inhibit cysteine proteases (Parks *et al.*, 1985). Two such inhibitors N-acetyl-leu-leu-nleu aldehyde (calpain inhibitor 1) and N-acetyl-leu-leu-methionine aldehyde (calpain inhibitor 2) (Sasaki *et al.*, 1990) were tested. Dose-response curves (Figure 2) show that, as with TPCK and ZPCK, both agents were effective at slowing the degradation of β and δ. The relative activity of these inhibitors was the same for δ and β; however, the graph shows that N-acetyl-leu-leu-nleu aldehyde was ~10-fold more potent than the N-acetyl-leu-leu-methioninyl derivative. The microbial peptidyl epoxide E64 is a potent and specific inhibitor of cysteine proteases (Barrett *et al.*, 1982). Surprisingly, prolonged incubation of cells with high concentrations (500 μg/ml) of E64 had no effect on degradation of either chain. Many studies have shown that E64 is inefficient at inhibiting intracellular proteases because it passes poorly across membranes. The cell permeability of E64 can be increased if the peptide is linked to hydrophobic side chains, and the effects of one

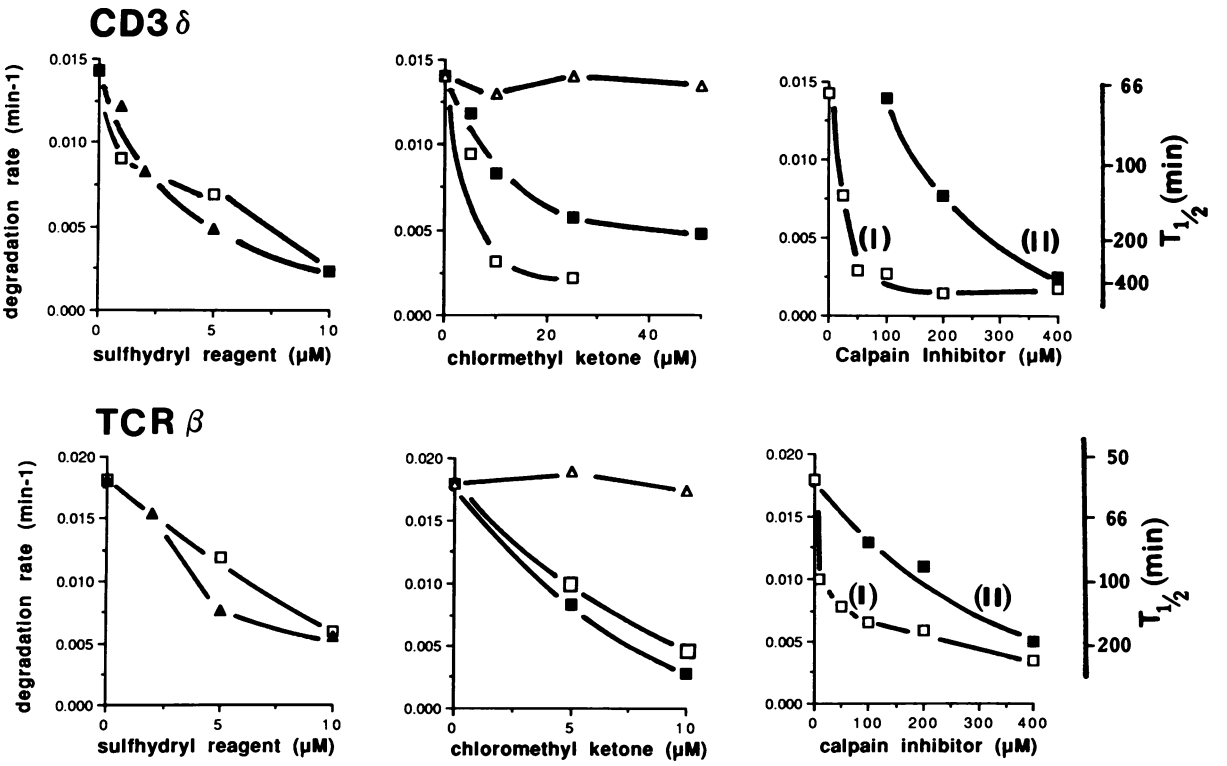

Figure 2. **Inhibitors of cysteinyl proteases slow degradation of TCR β and CD3 δ.** CHO cells transfected with TCR β or CD3 δ were incubated at 37°C in HEPES-buffered RPMI, pH 7.4, for 2 h with the indicated concentration of inhibitor. Cells were washed and then warmed to 37°C in the same concentration of inhibitor in HEPES-buffered RPMI containing cycloheximide. Cell aliquots (10^6) were taken at suitable time points (chosen to cover the degradation of at least one half of the subunit) and assayed for the presence of transfected protein using the subunit-specific ELISAs. The rate of degradation was calculated from linear portions of degradation curves (see panel B of Figure 1). The top panel shows an analysis of CD3 δ and the lower panel shows an analysis of TCR β. Sulphydryl reagents: (▲) N-ethylmaleimide and (□) iodoacetamide. Chloromethyl ketones: (△) TLCK, (■) TPCK, and (□) ZPCK. Calpain inhibitors: (□) calpain inhibitor 1 and (□) calpain inhibitor 2.

such derivative, E64-D (McGowan *et al.*, 1989), on degradation was tested. Figure 3 compares the effects of E64 and E64-D on degradation of CD3 δ. E64-D, which contains an extra ethyl group, was able to inhibit degradation. These results again pointed to the activity of cysteine proteases as being important components of the degradation pathway.

Inhibitors of degradation do not inhibit the action of cycloheximide

A central requirement of the cycloheximide-chase ELISA assay for degradation is that protein synthesis has to be stopped before pre-synthesized β and δ chains can be chased into the degradative pathway. A possible artifact could arise in these experiments if the sulfhydryl reagents and chlormethyl ketones were, in some unpredictable manner, able to inhibit the action of cycloheximide on protein synthesis. To ensure that this was not the case, the effects of the protease inhibitors on the ability of cycloheximide to stop protein synthesis was tested. CHO cells were preincubated with the protease inhibitors for 2 h and then pulse-labeled with ^{35}S-methionine and cysteine for 10 min in the presence or absence of cycloheximide. Cell lysates were precipitated with trichloroacetic acid and soluble radioactivity was removed by washing on glass filters. The levels of incorporation of radiolabel into protein are shown in Table 2. As expected, cycloheximide reduced the incorporation of ^{35}S-methionine into trichloroacetic acid insoluble protein. Most importantly this inhibition of protein synthesis was not reversed by the protease inhibitors that slowed degradation of TCR β and δ chains.

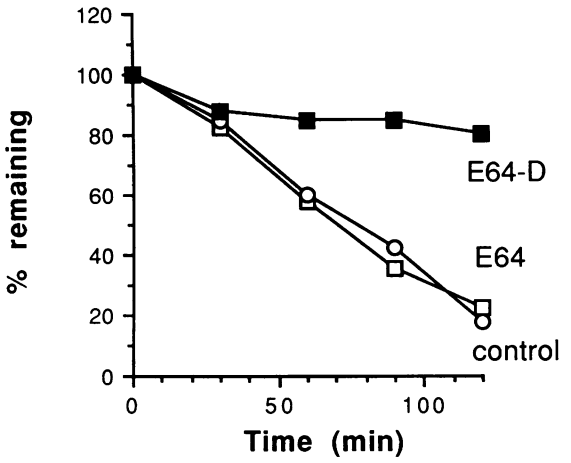

Figure 3. **Effect of E64 and E64-D on degradation of CD3 δ.** CHO cells transfected with CD3 δ were incubated with E64 or E64-D (400 μg/ml) in HEPES-buffered RPMI, pH 7.4, for 2 h at 37°C. Cells were washed and warmed to 37°C in the same concentration of inhibitor in HEPES-buffered RPMI containing cycloheximide. Cell aliquots (10^6) were taken at the indicated time points and assayed using the δ specific ELISA. (○) control, (□) E64, (■) E64-D.

Inhibitors of degradation do not affect ATP production and do not inhibit constitutive secretion

It was predicted that sulphydryl reagents, chloromethyl ketones, and peptidyl aldehydes could have a range of effects on cell metabolism and that these pleiotrophic effects, rather than inhibition of protease activity, could be responsible for the slowed degradation caused by these compounds. Trypan blue was used as a preliminary screen of toxicity and at the concentrations of inhibitors used, cells were able to exclude Trypan Blue during the course of the experiments. To assess the level of specificity of these compounds at a higher level, their effect on the production of ATP was studied as a means of assessing their effects on cellular metabolism. Cell aliquots were taken during the course of each experiment and assayed for ATP using a luciferin/luciferase assay. Table 3 shows the effects of the protease inhibitors on cellular ATP levels and compares these values with those found in cells incubated in glucose-free media and 2-deoxyglucose. Table 3 shows that incubation of cells at 37°C for 30 min with the 50 mM 2-deoxyglucose in glucose-free RPMI caused a fall in cellular ATP to 15–20% of control (28 nM/mg protein). Significantly, the sulfhydryl reagents, chlormethyl ketones, and calpain inhibitors that had a marked effect on degradation (Figure 2) were well tolerated by cells and caused little perturbation in ATP levels. ZPCK caused a small fall in cellular ATP to 78% of control, whereas 1,10-phenanthroline lowered ATP levels to 65% of control. Table 3 shows also that degradation itself was not highly dependent on ATP. Cells treated with 50 mM 2-deoxyglucose and 1 mM NaCN maintained ATP levels that were only 5% of control cells

Table 2. Effect of protease inhibitors on cycloheximide function and protein synthesis

Inhibitor	Concentration	^{35}S-Met Incorporation (cpm/5 × 10^6 cells)
Control		74 000
Cycloheximide	10 μg/ml	7 200
NEM	5 μM	1 850
Iodoacetamide	5 μM	3 100
TPCK	10 μM	2 500
ZPCK	10 μM	1 100
Calpain inhibitor I	100 μM	3 600
Calpain inhibitor II	400 μM	2 500
1,10-Phenanthroline	500 μM	850

CHO cells were preincubated with the protease inhibitors as described in the legend to Figure 2. Cells were then pulse-labeled for 10 min with ß^{35}S-methionine in the presence of 10 μg/ml cycloheximide and indicated protease inhibitor. Cells were then precipitated with 15% trichloroacetic acid. Precipitates were collected on filters washed, and counted. The table compares methionine incorporation under these conditions with control cells incubated in the absence of cycloheximide or protease inhibitor. NEM, N-ethylmaleimide.

Table 3. Cellular ATP levels after incubation with protease inhibitors

Inhibitor	Concentration	ATP (% control)	Half-life (min) δ	Half-life (min) β
None		100	58	60
2-deoxyglucose	50 mM	15	53	55
NaCN +2-deoxyglucose		5	56	60
Monensin	10 μM	68	60	60
Iodoacetamide	5 μM	110	125	110
N-ethylmaleimide	5 μM	115	180	175
TPCK	10 μM	105	288	210
ZPCK	10 μM	78	120	220
Calpain 1	100 μM	123	430	180
Calpain 2	400 μM	118	390	200
E64	500 μg/ml	58	60	60
E64-D	500 μg/ml	107	300	nd
Phenanthroline	500 μM	65	410	380

CHO cells were incubated for 3 h at 37°C with the indicated concentrations of protease inhibitors dissolved in HEPES-buffered RPMI, pH 7.4. Cells were washed and warmed to 37°C in fresh media containing the inhibitor. One hour later cell aliquots were taken and assayed for ATP using a luciferin/luciferase bioluminescence assay. The results presented were adjusted for total cellular protein content.

yet were able to degrade TCR β and CD3 δ as rapidly as untreated control cells.

CHO cells transfected with an "anchor minus" interleukin 2 (IL2) receptor secrete the ectodomain of the receptor constitutively into the culture medium (Treiger *et al.*, 1986). It was anticipated that an analysis of protein secretion by such cells would be a good indicator of the function of the endoplasmic reticulum and also would be a monitor of the effects of cysteine protease inhibitors on N-ethylmaleimide sensitive factor (NSF). NSF is a cytosolic protein that catalyzes the many fusion events that take place during the transport of vesicles along the secretory pathway and, as its name suggests, is sensitive to inactivation by sulphydryl reagents. CHO cells transfected stably with the truncated IL2 receptor were incubated with concentrations of protease inhibitors that had been shown in Figure 2 to cause a near complete inhibition of degradation. The effect of the inhibitors on the accumulation of IL2 receptor epitopes in the culture media (Figure 4) was taken as a measure of their effect on constitutive secretion. The data at the head of Figure 4 show that two control compounds, brefeldin-A and monensin, that have a well-documented ability to inhibit constitutive secretion (Tartakoff, 1983; Fujiwara *et al.*, 1988; Lippincott-Schwartz *et al.*, 1989), were able to markedly slow the secretion of IL2 receptor epitopes into the culture medium. Figure 4 also shows that the peptidyl chloromethyl ketones and calpain inhibitors that slowed degradation had little effect on secretion. For example, at concentrations that produced a four- to sixfold decrease in the rate of proteolysis, N-ethylmaleimide, TPCK, ZPCK, the calpain inhibitors, and E64-D slowed secretion of IL2 receptor epitopes only 20–30%. 1,10-Phenanthroline produced a 50% fall in the rate of secretion. Taken together the results show that cysteine protease inhibitors slow degradation in the endoplasmic reticulum without adversely affecting the ability of the organelle to deliver newly synthesized protein into the secretory pathway. Furthermore, these compounds do not affect the transport of these proteins through the Golgi to the cell surface.

Inhibitors of cysteine proteases do not affect disulphide bond formation in the endoplasmic reticulum

Protein disulphide isomerase is an abundant resident protein of the lumen of the endoplasmic reticulum. The enzyme is thought to aid the correct folding and assembly of newly synthesized proteins through its ability to catalyze the rearrangement of disulphide bonds (Bullied and Freedman, 1988). The susceptibility of newly synthesized proteins to proteolysis within the endoplasmic reticulum may well be determined by the conformation that proteins adopt after

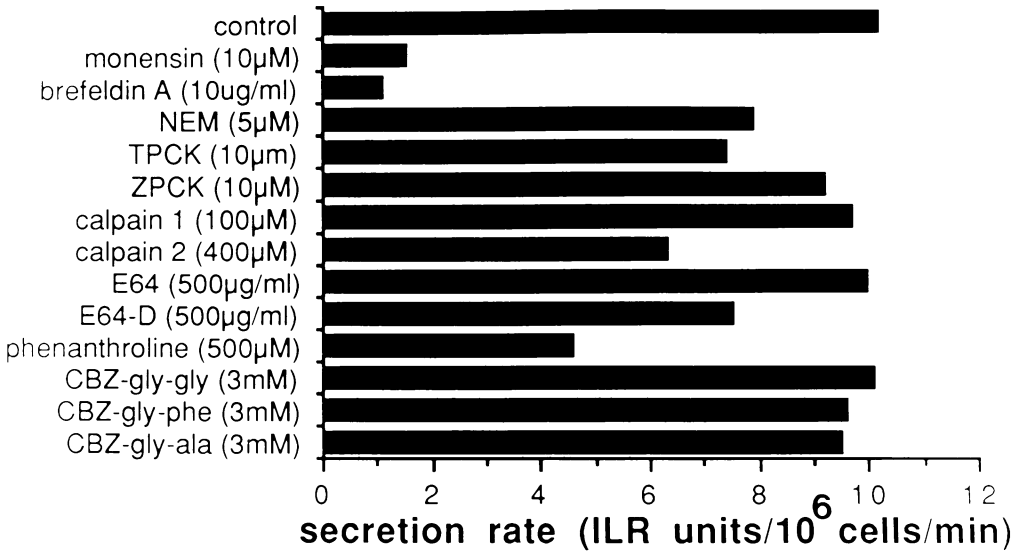

Figure 4. **Effect of protease inhibitors on constitutive secretion from CHO cells.** CHO cells transfected stably with the ectodomain of the IL2 receptor were grown to confluence on 6-well plates. Cells were preincubated with the indicated concentration of protease inhibitor for 2 h at 37°C. Cells were then washed and fresh HEPES-buffered RPMI containing the indicated concentration of inhibitor was added. One hundred-microliter aliquots of culture supernatant were taken over the subsequent 3 h and assayed for secreted IL2 receptor epitopes using an ELISA. The graph shows the calculated rates of secretion under each condition. Brefeldin A was added 10 min and monensin was added 30 min before the collection of supernatants.

synthesis, and interchain disulphide bond formation may be an important determinant of their ability to be recognized as substrates for proteolysis. In the context of the experiments reported above, it is possible that the inhibition of degradation of TCR β and CD3 δ observed in cells incubated with inhibitors of cysteine proteases may have resulted from an action of the reagents on the free sulphydryl groups of the newly synthesized chains. The observed inhibition of proteolysis need not necessarily reflect the inactivation of a cysteine protease within the endoplasmic reticulum. The ζ chain of the TCR forms a disulphide-linked homodimer soon after synthesis. CHO cells transfected stably with the human ζ chain were used to see if the protease inhibitors could affect the formation of a disulphide-linked ζ dimer. The cells were incubated for 2 h at 37°C with concentrations of protease inhibitors that were shown in Figure 2 to be able to inhibit degradation of TCR β and CD3 δ. Cells were then pulse-labeled with ^{35}S-methionine and cysteine and immunoprecipitated with a rabbit antiserum (N 39) that reacts with the C-terminus of the protein. Immunoprecipitated protein was analyzed by non-reducing SDS-PAGE. Each lane of the gel shown in Figure 5 resolves two immunoprecipitated proteins. The most abundant band seen at 32 kDa is the disulphide-linked ζ dimer. The ζ monomer (16 kDa) can be seen as the minor band migrating toward the bottom of the gel. The relative levels of ζ monomer and disulphide-linked ζ dimer were not changed when cells were incubated with the indicated protease inhibitors. The results suggested that at the concentrations used the inherent sulphydryl reactivities of the protease inhibitors were not at a level that could modify the free sulphydryl groups of newly synthesized ζ chains. The results again suggest that degradation is inhibited through the specific modification of a cysteine protease.

Discussion

In this report we have taken the simple approach of incubating cells with protease inhibitors as a means of characterizing the proteases that degrade proteins in or close to the endoplasmic reticulum. We have used CHO cells transfected with either TCR β or CD3 δ and a rapid ELISA-based assay to study degradation. TCR β and CD3 δ have widely different amino acid sequences (Van den Elsen *et al.*, 1984; Yanagi *et al.*, 1984), and this has allowed the study to document the effects of protease inhibitors on the degradation of two different substrates for pre-Golgi proteolysis. The results show that, of the many protease inhibitors tested, inhibitors

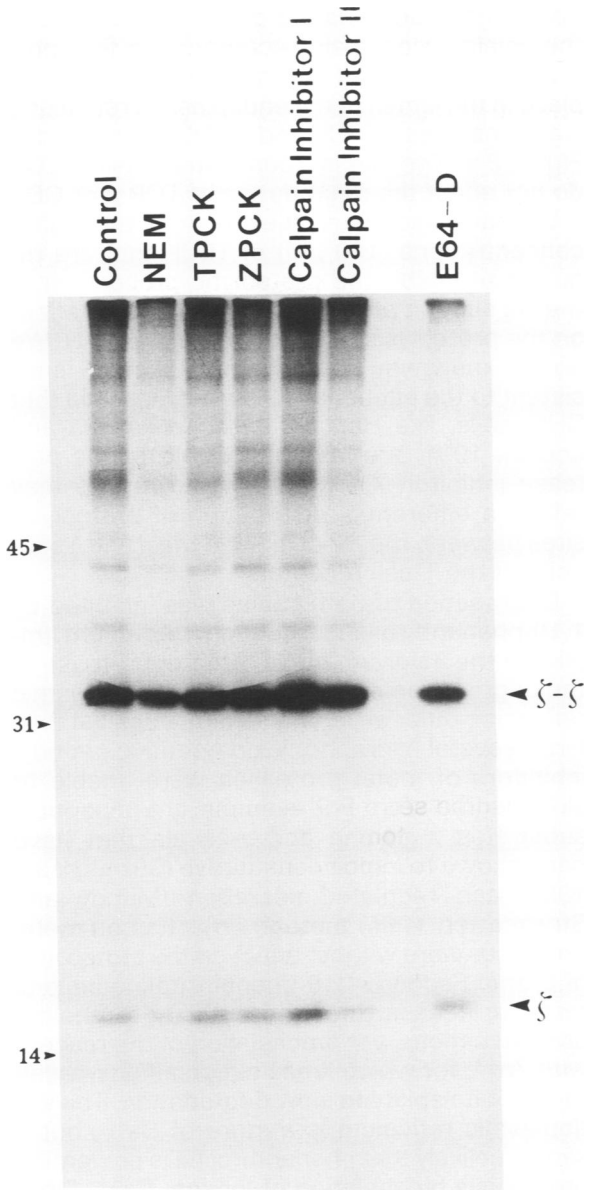

Figure 5. **Effect of protease inhibitors on synthesis and assembly of a disulphide-linked TCR ζ homodimer.** CHO cells were transfected stably with the human TCR ζ chain. Cells were preincubated with the indicated protease inhibitor for 2 h at 37°C and then pulse-labeled with ^{35}S-methionine and cysteine in the presence of the protease inhibitor for 1 h at 37°C. Cells were lysed using 1% NP 40 and immunoprecipitated using a rabbit polyclonal antiserum specific for c-terminal amino acids 131–141 of the ζ chain. Immunoprecipitates were analyzed by 12.5% SDS-PAGE under nonreducing conditions. The positions of molecular weight markers (kD) are shown as are the TCR ζ monomer and disulphide linked homodimer.

of cysteine proteases consistently slowed degradation of both TCR β and CD3 δ. Figure 2 presents dose-response curves for the effects of these compounds and shows that degradation was inhibited by micromolar concentrations of the sulfhydryl modifying reagents, N-ethylmaleimide and iodoacetamide, and by phenylalanyl chloromethyl ketones. Inhibitors of calcium-activated cysteine proteases (calpains) were also effective, as was E64-D, a hydrophobic analogue of E64, a peptidyl epoxide inhibitor of cysteine proteases.

Although the chloromethyl ketone, aldehyde, and epoxide inhibitors listed above were synthesized to react with the cysteine residues at the active site of cysteine proteases, they also have the potential to react with thiol groups in general (reviewed by Shaw, 1990). In view of this it was possible that the inhibition of degradation in the endoplasmic reticulum produced by this class of inhibitor may have been secondary to effects of these compounds on the thiol groups of proteins other than cysteine proteases within the cell. A series of controls were incorporated into the study, and these tested the effects of the protease inhibitors on cellular metabolism, constitutive secretion, and disulphide bond formation in the endoplasmic reticulum. Essentially very little evidence for nonspecific effects was revealed by these assays. In particular it was anticipated that an analysis of secretion rates would provide a good indicator of effects arising from nonspecific modification of thiol groups. The secretion of protein from cells requires the action of a cytosolic protein, NSF, that is itself inactivated by sulphydryl reagents. NSF catalyses the fusion of transport vesicles with their target organelles as they pass their contents sequentially from the endoplasmic reticulum to the Golgi apparatus and then to the plasma membrane (Mulhotra *et al.,* 1988; Wiedman *et al.,* 1989). When added to CHO cells at concentrations that produced a near complete inhibition of degradation of TCR β or CD3 δ, N-ethylmaleimide, ZPCK, TPCK, the dipeptidyl aldehyde calpain inhibitors, and E64-D at most produced a 20% inhibition of secretion. The experiment showed that secondary effects arising from nonspecific modification of sulfhydryl groups by the protease inhibitors were not at a level that could significantly disrupt the activity of cytosolic NSF. In a second series of experiments the effects of the protease inhibitors on cellular ATP levels were tested as a measure of their effects on cellular metabolism. Table 3 shows that concentrations of cysteine protease inhibitors that effectively slowed degradation did not cause a major fall in cellular ATP levels, and it is concluded that they were not generally toxic to cells. Finally,

experiments were conducted to assess the effects of the cysteine protease inhibitors on disulphide bond formation within the endoplasmic reticulum. The results presented in Figure 5 showed that the ability of the endoplasmic reticulum of CHO cells to synthesize and assemble a disulphide-linked ζ dimer was not impaired by incubation with the protease inhibitors.

Although the results show a sensitivity of degradation in the endoplasmic reticulum to cysteine protease inhibitors, they do not discount the involvement of other classes of proteases. One reason for this is that the assay employed in this study uses whole cells, and not surprisingly, all the inhibitors of degradation described above were hydrophobic. For a protease inhibitor to be effective it has to reach the site of proteolysis, and in the case of degradation of the CD3 δ and TCR β chains, this requires movement of the inhibitor across the plasma membrane and the limiting membrane of the endoplasmic reticulum. Cysteine proteases have a preference for hydrophobic amino acids such as phenylalanine and as a consequence many cysteine protease inhibitors are themselves hydrophobic (Shaw, 1990). It is significant that many studies have now shown that degradation in the endoplasmic reticulum is resistant to the effects of hydrophilic peptide protease inhibitors such as leupeptin, pepstatin, and E64 (Chen *et al.,* 1988; Lippincott-Schwartz *et al.,* 1988; Amara *et al.,* 1989; Bonifacino *et al.,* 1989; Wileman *et al.,* 1990b). Such results suggest that the proteolytic compartment associated with the endoplasmic reticulum is inaccessible to hydrophilic molecules that are unable to cross membranes efficiently. The results using E64 and the hydrophobic analogue E64-D illustrate this point. Although high concentrations of E64 were without effect on degradation, the hydrophobic analogue E64-D, which is able to cross membranes (McGowan *et al.,* 1989), was able to slow degradation of CD3 δ. Assays for proteolysis that use whole cells, or those employing "broken cells" where the membrane of the endoplasmic reticulum remains intact, are likely to favor the identification of hydrophobic protease inhibitors. Interestingly, not all the hydrophobic protease inhibitors that were tested had an effect on degradation. Two sparingly soluble inhibitors of serine proteases, PMSF and 3,4-dichloroisocoumarin, were without effect on proteolysis of TCR β or CD3 δ (Table 1).

Recently, Wikstrom and Lodish (1991) have followed the effect of a panel of protease inhibitors on the degradation of the H2 subunit of the asialoglycoprotein receptor. The first proteolytic cleavage of the subunit, which takes place in the lumen of the endoplasmic reticulum, was sensitive to TPCK and TLCK. Interestingly, Figure 2 shows that low concentrations of TLCK do not affect the degradation of TCR β or CD3 δ. We have also tested the effects of the higher concentrations (100 μM) of TLCK that are required to inhibit the proteolytic processing of the H2 subunit and have again seen little effect on the proteolysis of TCR β and δ (Table 1). We do not know why degradation of β and δ is resistant to the effects of TLCK. It is possible that CHO cells, like Hep G2 cells, (Wikstrom and Lodish, 1991) are less permeable to the protease inhibitor. Alternatively, the results may reflect a differential distribution of proteolytic sites between the H2 chain and the β and δ subunits of the T-cell receptor.

Degradation of both chains was inhibited by 1,10-phenanthroline. Although this might implicate the involvement of a metal protease in the degradation of proteins in the endoplasmic reticulum, other experiments argue against this. First, several hydrophobic competitive peptidyl inhibitors of metal proteases were unable to slow degradation. For example, the dipeptidyl substrates z-gly-phe and z-gly-ala that have been shown to inhibit constitutive (Strous *et al.,* 1988) and regulated secretion (Mundy and Strittmatter, 1985) through an action on metal proteases were without effect on the proteolysis of β or δ. Second, 1,10-phenanthroline inhibits metal proteases through its ability to chelate divalent cations, yet prechelation of the reagent with Zn^{++}, for which it has a high affinity, failed to affect its ability to slow degradation. The endoplasmic reticulum is a store of Ca^{++}, but it seems unlikely that phenanthroline is preventing proteolysis by chelation of luminal Ca^{++}. First, 1,10-phenanthroline has a very low affinity for Ca^{++}, and prechelation of 1,10-phenanthroline with equimolar concentrations of Ca^{++} did not reduce its effects on degradation. Second, depletion of calcium from the endoplasmic reticulum has been shown to increase, not decrease, the rate of degradation of TCR β and CD3 δ (Wileman *et al.,* 1991). In summary, 1,10-phenanthroline is undoubtedly an inhibitor of pre-Golgi degradation, but its precise mode of action remains unclear.

In this study we have shown that inhibitors of cysteine proteases consistently slow the degradation of newly synthesized chains of the TCR. They act in a dose-dependent manner and

inhibit degradation at concentrations that do not appear to affect cellular ATP levels, constitutive secretion by cells, or disulphide bond formation. These results implicate cysteine proteases as being at least one class of protease that are involved in the removal of misfolded or partially assembled proteins from the endoplasmic reticulum. One likely interpretation of the results would be that cysteine proteases themselves act on and degrade proteins within the endoplasmic reticulum. It is not possible, however, to exclude other possibilities. For example, the action of a cysteine protease may be required for the transport of β and δ from their site of synthesis to their site of degradation. Alternatively, it is possible that the cysteine protease inhibitors have a high affinity for some other sulphydryl group that is crucial for the proteolysis of TCR β and CD3 δ chains. Further experiments will be required to resolve these points. In the meantime the identification of sulphydryl reagents and inhibitors of cysteine proteases as being consistent inhibitors of "pre-Golgi" degradation should allow them to become useful tools for further studies on the selective degradation of newly synthesized proteins.

Materials and methods

TPCK, TLCK, N-acetyl-leu-leu-nleu aldehyde (calpain inhibitor 1), N-acetyl-leu-leulmethionine aldehyde, and L-3-trans-2,3-epoxypropionyl-leucylamido-(4-benzyloxy-carbonylamino)butane (E64) were purchased from Boehringer Mannheim (Indianapolis, IN). 1,10-Phenanthroline, ZPCK, 3,4,dichloroisocoumarin, pepstatin and chymostatin, the carbobenzoxy dipeptides, and luciferin/luciferase assay kits were purchased from Sigma (St. Louis, MO). Phenylbutyryl-leu-nleu-chloromethyl ketone was a generous gift from Dr. Naoki Higuchi of Suntory Limited (Osaka, Japan). E64-D was a generous gift from Dr. K. Handa of Taiso Pharmaceuticals (Omiya, Japan). Brefeldin A was purchased from Epicenter Technologies (Madison, WI).

Cells and transfections

The CHO cell lines K1 (ATCC# CCl 61) and DUX B11 (dhfr$^-$ CRL 9010) were obtained from American Type Culture Collection (Rockville, MD). The K1 CHO cells were maintained in 5% CO_2 at 37°C in a base medium of RPMI supplemented with glutamine (2 mM) and 8% heat-inactivated fetal calf serum. DUX B11 cells were grown in the same medium supplemented with 15 μg/ml proline. Tissue culture media were purchased from GIBCO (Grand Island, NY) or Sigma. The transfection of cells with the TCR β chain and CD3 δ has been reported previously (Wileman *et al.*, 1990a,b) as has the construction and transfection of the "anchor minus" mutant of the IL2 receptor (Wileman *et al.*, 1990c). The c-DNA encoding the human TCR ζ chain was subcloned into the *Xho*I site of the expression vector pMNSXND (Lee and Nathans, 1988). CHO DUX B11 cells were transfected with calcium phosphate precipitates of the vector. Stable clones were isolated after selection in base media containing 500 μg/ml G418 (GIBCO).

Antibodies

W76 and IdentiTy β-F1$^{(TM)}$ were provided by T-Cell Sciences (Cambridge, MA) and are specific for TCR β. SP 19 and SP 64 precipitate CD3 δ (Pessano *et al.*, 1985). For use in ELISAs the antibodies were affinity purified from ascites using Protein G Sepharose (Pharmacia, Piscataway, NJ). β-F1 and SP 64 were coupled to periodate-oxidized sugars of horseradish peroxidase. N39 is a rabbit polyclonal antibody raised against a C-terminal peptide of the human TCR ζ chain.

Metabolic labeling, immunoprecipitation, and electrophoresis

Transfected cells were labeled metabolically with ^{35}S-methionine and cysteine using Trans ^{35}S (ICN Radiochemicals, Cleveland, OH). Cells (90% confluent 10-cm dish) were preincubated in methionine and cysteine-free media for 30 min, washed, and then pulse-labeled for 30 min at 37°C. Cells were washed and then chased for the indicated time intervals in complete media. Labeled cells were lysed at 4°C in immunoprecipitation buffer (10 mM ethanolomine, pH 7.8, containing 1% NP 40, 0.15 M NaCl, 10 mM iodoacetamide, 1 mM EDTA, 1 mM PMSF, and 1 μg/ml of the following protease inhibitors: leupeptin, pepstatin, chymostatin, and antipain). Lysates were clarified by centrifugation, precleared, and immunoprecipitated as described previously (Wileman *et al.*, 1990a). PAGE was performed using 12.5% slab gels. Gels were fixed and incubated in 1 M salicylate before autoradiography.

ELISA for TCR β, CD3 δ, and the IL2 receptor

The capture antibodies (W76 for TCR β and SP 19 for CD3 δ) were applied to Immulon 96 well plates (Dynatec Labs. Inc., Chantilly, VA) as 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. Antigen samples, diluted to 200 μl with blocking buffer, were applied to drained wells and incubated for 2 hours at 37°C. Bound antigen was visualized in washed wells using horseradish peroxidase-coupled 2nd antibodies (β F1 for β and SP 64 for δ) diluted in PBS containing 30% fetal calf serum, 6% PBS, and 0.75% NP40. Bound peroxidase was determined colorimetrically by addition of o-phenylenediamine and H_2O_2. IL2 receptor epitopes were analyzed using the Cell free^{tm} IL2R assay kit from T-Cell Sciences.

Cycloheximide-chase ELISA

The details of this assay have been described previously (Wileman *et al.*, 1990b). CHO cells transfected with either TCR β or CD3 δ were adapted to suspension culture and grown in spinner flasks. Unless otherwise indicated, cells were tumbled (10^6/ml) at 37°C for 2 hours in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered RPMI (pH 7.4) containing the stated concentration of protease inhibitor. Cells were then pelleted and cooled to 4°C. To start the chase, cells were warmed to 37°C by adding HEPES-buffered RPMI (pH 7.4) containing the protease inhibitor and 10 μg/ml cycloheximide. At suitable time points,

cells (10^6) were pelleted and lysed in 300 μl of 10 mM ethanolamine buffered to pH 7.8 containing 1% NP40, 1 mM EDTA, 1 mM PMSF, and 1 μg/ml of the following protease inhibitors: leupeptin, pepstatin, chymostatin, and antipain. Lysates were left on ice for 1 h, insoluble material was pelleted by centrifugation, and supernatants (100-μl aliquots) were assayed for antigen using the relevant ELISA.

Estimation of ATP

ATP concentrations were assayed using an ATP bioluminescence assay kit from Sigma. Luminescence was measured using a Lumat LB 9501 luminometer (Berthold Instruments, Wildbad, Germany). Cellular protein was estimated using the BioRad protein assay (BioRad, Richmond, CA).

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