A Permeabilized Cell System Identifies the Endoplasmic Reticulum as a Site of Protein Degradation

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Abstract. Analysis of the fate of a variety of newly synthesized proteins in the secretory pathway has provided evidence for the existence of a novel protein degradation system distinct from that of the lysosome. Although current evidence suggests that proteins degraded by this system are localized to a pre-Golgi compartment before degradation, the site of proteolysis has not been determined. A permeabilized cell system was developed to examine whether degradation by this pathway required transport out of the ER, and to define the biochemical characteristics of this process. Studies were performed on fibroblast cell lines expressing proteins known to be sensitive substrates for this degradative process, such as the chimeric integral membrane proteins, Tac-TCRα and Tac-TCRβ. By immunofluorescence microscopy, these proteins were found to be localized to the ER. Treatment with cycloheximide resulted in the progressive disappearance of intracellular staining without change in the ER localization of the chimeric proteins. Cells permeabilized with the pore-forming toxin streptolysin O were able to degrade these newly synthesized proteins. The protein degradation seen in permeabilized cells was representative of that seen in intact cells, as judged by the similar speed of degradation, substrate selectivity, temperature dependence, and involvement of free sulfhydryl groups. Degradation of these proteins in permeabilized cells took place in the absence of transport between the ER and the Golgi system. Moreover, degradation occurred in the absence of added ATP or cytosol, and in the presence of apyrase, GTPγS, or EDTA; i.e., under conditions which prevent transport of proteins out of the ER. The efficiency and selectivity of degradation of newly synthesized proteins were also conserved in an isolated ER fraction. These data indicate that the machinery responsible for pre-Golgi degradation of newly synthesized proteins exists within the ER itself, and can operate independent of exogenously added ATP and cytosolic factors.

PROTEIN degradation pathways are essential for the maintenance of cellular homeostasis and participate in a vast range of biological processes. Even though virtually all cellular compartments contain proteolytic enzymes, the major sites of protein breakdown within eukaryotic cells are the cytosol and lysosomes (see reviews by Bond and Butler, 1987; Rechsteiner, 1987). Recent studies on the fate of newly synthesized integral membrane and lumenal proteins have provided evidence for the existence of a novel degradative system in the early secretory pathway. A variety of proteins that fail to be transported into the Golgi system because of improper folding or lack of assembly into oligomers have been found to be actively degraded by a process referred to as "pre-Golgi degradation" or "ER degradation," (reviewed by Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991).

Perhaps the most comprehensive description of the characteristics of this degradative pathway has emerged from the study of the multisubunit T cell antigen receptor (TCR)[1] complex (reviewed by Klausner et al., 1990). This receptor is composed of at least seven chains ($\alpha\beta\gamma\delta\epsilon\zeta_2$), which must be assembled in the ER for efficient transport to the cell surface. Unassembled chains or partially assembled complexes in most cases are selectively degraded (Lippincott-Schwartz et al., 1988; Chen et al., 1988; Bonifacino et al., 1989; Wileman et al., 1990*a*,*b*). Asparagine-linked carbohydrates of the TCR chains degraded by this pathway remain sensitive to endoglycosaminidase H (endo H), consistent with localization to a compartment proximal to the medial-Golgi before degradation. A variety of inhibitors of lysosomal degradation including acidotropic agents (i.e., chloroquine, NH_4Cl), specific protease inhibitors (i.e., leupeptin, E64), or amino acid methyl esters all have little or no effect on pre-Golgi degradation in vivo, suggesting that typical lysosomes are not involved in this process. A distinctive characteristic of the pre-Golgi degradation of TCR chains is its selectivity with respect to substrates. Several studies have shown that whereas some TCR chains (α, β, γ, and δ) can be rapidly destroyed by this process, others (ϵ and ζ) remain stable for long periods of time (Chen et al., 1988; Bonifacino et al., 1989; Wileman et al., 1990*a*,*b*). For some of the receptor

1. *Abbreviations used in this paper*: SLO, streptolysin O; TCR, T cell antigen receptor.

chains, sensitivity to degradation by this pathway has been found to be a result of the presence of specific structural information that determines their rapid degradation. Within the α and β chains of the TCR, these determinants have been localized to their single membrane-spanning domains (Bonifacino et al., 1990*a*,*b*; Wileman et al., 1990*c*).

A number of other unassembled subunits of oligomeric complexes, resident ER proteins, abnormal and even seemingly normal proteins have been shown to be degraded by a pathway with similar characteristics to that described for TCR chains. These include immunoglobulin heavy chains (Sidman, 1981; Dulis et al., 1982; Sitia et al., 1987), the H2a subunit of the asialoglycoprotein receptor (Amara et al., 1989), acetylcholine receptor subunits (Merlie and Lindstrom, 1983; Blount et al., 1990), 3-hydroxy-3 methylglutaryl coenzyme A reductase (Gil et al., 1985; Jingami et al., 1987; Chun et al., 1990), acetylcholinesterase (Rotundo, 1988), apolipoprotein B (Davis et al., 1990; Sato et al., 1990), a β lactamase-α globin chimera (Stoller and Shields, 1989) and mutant forms of influenza hemagglutinin (Doyle et al., 1986), the low density lipoprotein receptor (Esser and Russell, 1988), β-hexosaminidase (Lau and Neufeld, 1989), and α_1-antitrypsin (Le et al., 1990; Curiel et al., 1990). Mutant forms of the cystic fibrosis transmembrane conductance regulator found in most patients with this disease also appear to be retained and degraded in a pre-Golgi compartment (Cheng et al., 1990). Recent studies have suggested a role for this degradative pathway in the processing of endogenous antigens for presentation to T cells (Weiss and Bogen, 1991). Despite the growing number of examples of this type of degradation and its important role in the posttranslational control of protein expression in physiological and pathological states, this proteolytic pathway has been poorly characterized.

An important issue that so far has remained unresolved is the intracellular localization of the degradative system. Biochemical and morphological evidence indicates that substrates are localized to the ER before degradation (Lippincott-Schwartz et al., 1988; Chen et al., 1988). However, proteolysis could occur within the ER system itself or in a compartment distal to the ER, such as the Golgi system. The lack of specific inhibitors of the degradative process or of transport between the ER and the Golgi system in intact cells has precluded an unambiguous distinction between these two possibilities. To examine a possible requirement for transport between the ER and the Golgi system and to further define the biochemical characteristics of the degradative process, we have used permeabilized cells in which traffic between organelles is disrupted. Cells permeabilized with the pore-forming toxin, streptolysin O (SLO), are shown to degrade proteins retained in the ER with the same general characteristics and selectivity displayed by intact cells. Degradation in the permeabilized cells occurs in spite of a total absence of transport between the ER and the Golgi system, and is independent of exogenously added ATP or cytosolic factors. Furthermore, agents known to disrupt transport between the ER and the Golgi system as well as other membrane transport pathways, such as apyrase, EDTA, and GTPγS, all fail to inhibit protein degradation. Taken together, these findings strongly suggest that degradation occurs within the ER system itself. This suggestion is confirmed by the observation that newly synthesized transmembrane proteins can be rapidly and selectively degraded in vitro in a subcellular fraction enriched in rough ER.

Materials and Methods

Recombinant DNAs

Complementary DNA encoding the Tac antigen (α chain of the human interleukin-2 receptor; Leonard et al., 1984) was the gift of Dr. Warren Leonard (National Institute of Child Health and Human Development). Construction of DNAs encoding the chimeric proteins Tac-TCRα_1 (hereon referred to as Tac-TCRα) and Tac-TCRβ has been described before (Bonifacino et al., 1990*a*,*b*; Manolios et al., 1990). All recombinant DNAs were cloned into the expression plasmid CDM8 (Seed, 1987), modified as previously described (Bonifacino et al., 1990*a*).

Transfections

The human fibroblast cell line M1 (a gift of Dr. Eric O. Long, National Institutes of Allergy and Infectious Diseases) was maintained in DME supplemented with 10% FBS and 0.15 mg/ml gentamicin (culture medium). For generation of stable transfectants, 3×10^5 cells plated on a 100-mm-tissue culture dish one day before transfection were incubated overnight (~12 h) with a calcium phosphate precipitate (Graham and Van der Eb, 1973; as modified by Gorman et al., 1983) containing 20 μg of a CDM8 plasmid and 2 μg of RSV.5(neo) (a gift of Dr. Eric O. Long) in 10 ml of culture medium. After incubation, cells were rinsed once with PBS, treated for 5 min with 10% DMSO in ice-cold PBS, rinsed once more with PBS, and incubated for 24 h in fresh culture medium. The medium was then replaced by fresh culture medium containing 0.5 mg/ml G418 (Gibco Laboratories, Grand Island, NY). The selective medium was changed every week. After 3–4 wk individual G418-resistant colonies were picked with sterile pipette tips and transferred to 48-well plates. Positive clones were identified by immunofluorescence microscopy, using anti-Tac antibodies (see below), and expanded in regular culture medium containing 0.25 mg/ml G418.

Antibodies

The mouse mAb 7G7, directed against an extracellular epitope of the human Tac antigen (Rubin et al., 1985a), was the kind gift of Dr. David Nelson (National Cancer Institute). The rabbit polyclonal antibody R3134, raised against purified human Tac (Sharon et al., 1986), and the mouse mAb anti-Tac were generously provided by Dr. Warren Leonard. The mouse mAb raised against human protein disulphide isomerase, HP13 (Kaetzel et al., 1987), was the generous gift of Dr. Charlotte Kaetzel (Case Western Reserve University, Cleveland, OH). Rhodamine-labeled donkey anti-rabbit and fluorescein-labeled donkey anti-mouse antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). These fluorescent antibodies had been passed through a solid-phase immunoadsorbent gel to remove antibodies which cross-react with immunoglobulins from other species, and were thus specifically prepared for double-labeling immunofluorescence experiments.

Specific Reagents

Reduced SLO was from Wellcome Diagnostics (Dartford, England). Apyrase Grade VI, iodoacetamide, creatine phosphokinase, phosphocreatine, and diamide were from Sigma Chemical Co. (St. Louis, MO). 2-deoxy-D-glucose, and adenosine-5'-triphosphate were from Calbiochem-Behring Corp. (La Jolla, CA). Leupeptin, aprotinin, hexokinase, and GTPγS were from Boehringer Mannheim Biochemicals (Indianapolis, IN). The lymphocytes from the enlarged lymph nodes of an MRL-*lpr*/*lpr* mouse were used as a convenient source of cytosol, which was prepared by the method of Beckers et al. (1987).

Biochemical Assays

ATP was measured using a diagnostic kit (Sigma Chemical Co.), which measured the conversion of ATP,3-phosphoglycerate, and NADH to glyceraldehyde-3-P, NAD, and phosphate. Using the luminometer from Analytical Luminescence Laboratory (Monolight 2010; San Diego, CA), the decrease in absorbance at 350 nm that resulted when NADH was oxidized to NAD was measured as an index of the amount of ATP present.

Lactate dehydrogenase (LDH) was measured using a colorimetric diagnostic kit (Sigma Chemical Co.). LDH converted pyruvic acid to lactic acid. An intensely colored phenylhydrazone was formed from residual pyruvic acid. The decrease in absorbance at 500 nm measured on a spectrophotometer (LKB Instruments, Inc., Gaithersburg, MD) was inversely proportional to LDH activity.

Metabolic Labeling

For the experiments on adherent cells depicted in Figs. 2 and 4, cells were grown in 100-mm-tissue culture plates, washed three times in methionine-free culture medium, and metabolically labeled by incubation in methionine-free culture medium containing 250 μCi/ml of [^{35}S]methionine (Tran ^{35}S-label; ICN Radiochemicals, Irvine, CA) for 15 min at 37°C. For experiments in intact cells, cells were washed with DME at 4°C, and placed in 5 ml regular culture medium at 37°C for the indicated chase periods. Cells were then scraped and separated from the medium by centrifugation for 5 min at 400 g. Both the cells and medium were then frozen at −70°C. For all subsequent experiments, cells grown attached to tissue culture flasks were put into suspension by incubating at 37°C for 5 min in Ca^{2+}- and Mg^{2+}-free HBSS with 5 mM EDTA added. Cells were then washed, and metabolically labeled as above. For experiments in intact cells, cells were washed with DME at 4°C, and resuspended in 1 ml regular culture medium at 37°C for the indicated chase periods. After centrifugation for 5 min at 400 *g*, the cell pellets were frozen at −70°C.

Cell Permeabilization

Permeabilization assay buffer consisted of 125 mM potassium acetate, 2.5 mM magnesium acetate, 10 mM glucose, 1 mM DTT, 25 mM Hepes, buffered to pH 7.2 with 1 M potassium hydroxide. After metabolic labeling, cells were washed once with permeabilization assay buffer at 0°C and incubated on ice for 15 min in 1.6 IU/ml SLO to allow binding of the polypeptide toxin. The excess toxin was washed off and the cells were incubated in assay buffer at 37°C for 5 min to allow polymerization of the toxin and pore formation. This protocol limits permeabilization to the plasma membrane, while it preserves the integrity of intracellular organelle membranes (Ahnert-Hilger et al., 1989). For the experiments on adherent cells depicted in Figs. 2 and 4, the cells were chased in 5 ml of assay buffer at 37°C. At the end of the chase period, the cells were scraped and they were separated from the assay buffer by centrifugation at 400 *g* for 5 min. The cells and supernatants were then frozen at −70°C. In subsequent experiments, cells in suspension were resuspended in permeabilization assay buffer and incubated at 37°C. 100 μl aliquots were then taken after the indicated chase periods and frozen at −70°C without separating cells from supernatant.

Preparation of a Rough ER Fraction

Subcellular fractionation was performed as described by Bole et al. (1986), and all steps were carried out at 0°C. After metabolic labeling, cells were washed in PBS and then washed and resuspended in 0.25 M sucrose in 5 mM Hepes buffered to pH 6.8. Cells were lysed in a nitrogen cavitation bomb by incubation at 50 psi for 15 min. After decompression the cells were disrupted with 20 strokes of a dounce homogenizer. Nuclei and intact cells were removed by centrifugation at 800 *g* for 10 min. The postnuclear supernatant was layered on top of a discontinuous sucrose gradient containing 1 ml:2 M, 3.4 ml:1.3 M, 3.4 ml:1 M, and 2.75 ml:0.6 M sucrose in 5 mM Hepes, pH 6.8, and centrifuged at 40,000 rpm in a rotor (model SW41; Beckman Instruments, Inc., Palo Alto, CA) for 2 h. The band at the 1.3 M:2 M interface was diluted with 0.25 M sucrose, 5 mM Hepes, pH 6.8, and centrifuged for 30 min at 50,000 rpm in a rotor (model 70.1/Ti; Beckman Instruments, Inc.). The pellet was resuspended in 400 μl permeabilization assay buffer and incubated at 37°C. After the indicated chase periods, 100 μl aliquots were frozen at −70°C.

Immunoprecipitation, Electrophoresis, and Autoradiography

Cells and supernatants were thawed at 0°C, and solubilized in ice-cold lysis buffer containing 0.5% (wt/vol) Triton X-100, 300 mM NaCl, 50 mM Tris-HCl (pH 7.6), and protease inhibitors, leupeptin (10 μg/ml), aprotinin (10 μg/ml), and iodoacetamide (10 mM). Insoluble material was removed by sedimentation at top speed in a microfuge (Beckman Instruments, Inc.) for 10 min at 4°C. The mAb, 7G7, adsorbed to protein A agarose (Bethesda Research Laboratories, Gaithersburg, MD) was then added to the supernatant for immunoprecipitation. After incubation for 1–2 h at 4°C, immunoprecipitates were washed five times in ice-cold saline buffer containing 0.1% (wt/vol) Triton X-100 to remove unbound proteins, and then washed once in ice-cold PBS. Bound antigen was eluted from the beads by boiling in SDS sample buffer containing 3% β-mercaptoethanol. Eluted proteins were analyzed by SDS-PAGE on 10% acrylamide gels, as described (Bonifacino et al., 1989). Fluorograms were scanned used a densitometer (Ultroscan XL; LKB Instruments Inc., Bromma, Sweden).

Immunofluorescence Microscopy

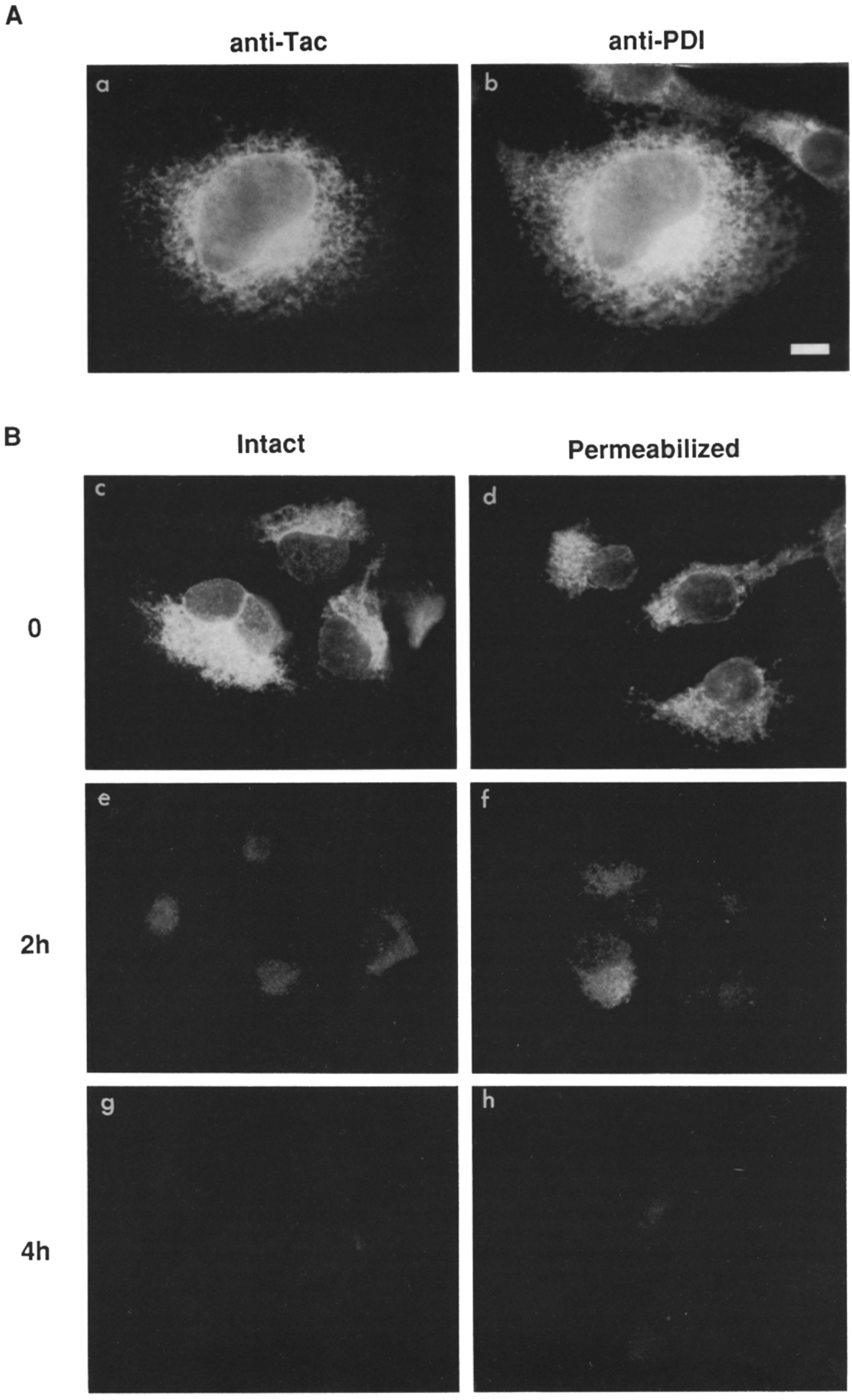
Cells were grown on 12-mm-round cover slips and fixed in 2% formaldehyde in PBS for 10 min at room temperature. Cells were washed in PBS/0.1% BSA, and then incubated for 1 h in antibody in PBS with 0.1% BSA and 0.1% saponin. After washing to remove unbound antibody, the cells were incubated with fluorescently labeled second antibody for 30 min. After a final wash in PBS, the cover slips were mounted on glass slides in Fluoromount G (Southern Biotechnology Associates, Birmingham, AL) and viewed with a microscope (Carl Zeiss Inc., Thornwood, NY) equipped with barrier filters to prevent crossover of fluorescein and rhodamine fluorescence.

Results

Immunofluorescent Staining Localizes a Rapidly Degraded Chimeric Protein to the ER

Degradation studies were performed on fibroblast cell lines expressing the chimeric protein Tac-TCRα, composed of the lumenal domain of the Tac antigen and the transmembrane and cytoplasmic domains of the TCR-α chain. This protein was previously shown to be rapidly degraded within a pre-Golgi compartment due to the presence of a specific determinant for degradation within the TCR-α transmembrane sequence (Bonifacino et al., 1990*a*,*b*). Immunofluorescence microscopy studies showed complete colocalization of Tac-TCR-α (Fig. 1 *a*) with protein disulphide isomerase (Fig. 1 *b*), a resident ER protein (Kaetzel et al., 1987). Both antibodies stained the nuclear envelope, as well as a fine reticular network extending throughout the cytoplasm, characteristic of the ER. The Tac-TCRα chimeric protein thus appeared to be confined to the ER system.

To follow the distribution of a cohort of Tac-TCRα from synthesis to degradation, and thus to determine whether degradation of Tac-TCRα was preceded by movement into another cellular compartment, cells were treated for variable periods before immunofluorescent staining with the protein synthesis inhibitor cycloheximide at 10 μg/ml. Consistent with the observed rate of degradation of the chimeric protein in these cells (see Fig. 2), fluorescent staining of Tac-TCRα was rapidly lost (Fig. 1, *c*, *e*, and *g*). At all time points before its disappearance, however, staining of Tac-TCRα was confined to a pattern typical of the ER. Recent studies have shown that certain proteins which are localized to the ER by morphological techniques can exit the ER and accumulate in a post-ER, intermediate compartment upon reduction in temperature from 16 to 20°C (Lippincott-Schwartz et al., 1990; Hsu et al., 1991). Treatment of cells expressing Tac-TCR-α at these reduced temperatures, however, resulted in no change in the immunofluorescent distribution of the chimeric protein (data not shown), suggesting that it is unable to exit the ER following this route. The data obtained from these immunofluorescent staining experiments suggested that Tac-TCRα did not exit the ER system to be degraded.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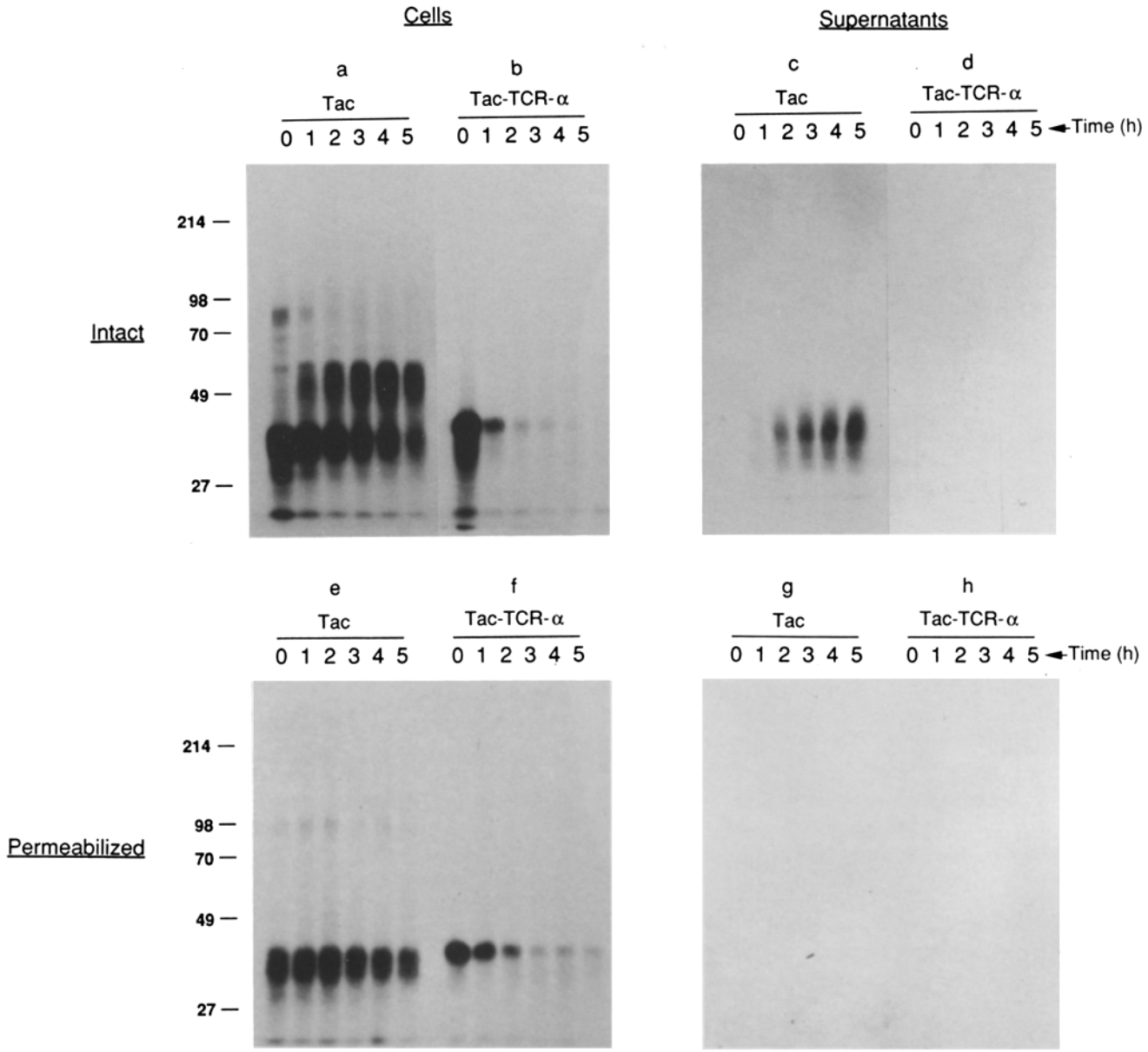

Figure 2. Rapid degradation of Tac-TCRα, but not Tac, in intact and permeabilized cells. M1 cells transfected with Tac or Tac-TCRα were pulse labeled for 15 min at 37°C with [^{35}S]methionine. Cells were left intact or permeabilized with streptolysin O and chased for the indicated time periods at 37°C in 5 ml medium or 5 ml assay buffer, respectively. Cells were removed from the plates, centrifuged at 2,000 rpm, and both pellets and supernatants were immunoprecipitated with the monoclonal anti-Tac antibody, 7G7, before analysis by SDS-PAGE.

Degradation of Tac-TCRα in Permeabilized Cells

To further examine whether transport out of the ER was required for degradation, a permeabilized cell system was developed. Fibroblasts stably transfected with DNA constructs encoding different protein substrates were permeabilized with SLO, as outlined in Materials and Methods. SLO is a pore-forming toxin synthesized by β-hemolytic group A streptococci. When added to cells it produces large, stable transmembrane pores with effective diameters exceeding 15 nm, that allow exchange of substances between the cytosol and the extracellular medium (Bhakdi and Tranum-Jensen, 1987). To limit permeabilization to the plasma membrane, cells were first incubated with SLO for 15 min at 4°C fol-

Figure 1. Immunofluorescent staining reveals that Tac-TCRα is confined to the ER system. (*A*) M1 cells expressing Tac-TCRα and permeabilized with saponin were stained with R3134, a rabbit polyclonal antibody to Tac (*a*), and HP13, a mouse mAb to protein disulphide isomerase (*b*), followed by rhodamine-labeled donkey anti-rabbit and fluorescein-labeled donkey anti-mouse antibodies. Specificity controls showed no cross-reactivity of the second with the first antibodies. (*B*) Intact (*c*, *e*, and *g*) or SLO-permeabilized (*d*, *f*, and *h*) M1 cells expressing Tac-TCRα were incubated at 37°C with 10 μg/ml cycloheximide for 0 (*c* and *d*), 2 (*e* and *f*), or 4 (*g* and *h*) h. Cells were then stained with R3134, followed by rhodamine-labeled donkey anti-rabbit antibody. Bar, 5 μm.

Table I. Consequences of Permeabilization of Cells with Streptolysin O

	Intact	Permeabilized
	%	%
Incorporation of Trypan blue (M_r = 960)	2	100
ATP (pmol) (M_r = 507)	21	1
Lactate dehydrogenase (B-B units)* (M_r = 135,000)	959	50

The amount of ATP and lactate dehydrogenase was measured in an equal number of intact and permeabilized cells, as described in Materials and Methods.
* *B-B units*, Berger-Broida units.

lowed by removal of the unbound toxin and an additional incubation for 5 min at 37°C (Bhakdi and Tranum-Jensen, 1987; Ahnert-Hilger et al., 1989). The characteristics of the cells permeabilized using this protocol are summarized in Table I. Permeabilization allowed incorporation of the dye Trypan blue (M_r = 960) in 100% of the cells, and resulted in escape of most of the cytosol, as evidenced by a 95% loss of ATP (M_r = 507), and a 95% loss of lactate dehydrogenase (M_r = 135,000). Mechanical permeabilization of cells has been shown to cause cessation of export from the ER due to the loss of ATP and cytosol (Beckers et al., 1987; Simons and Virta, 1987). To test whether SLO-permeabilized cells were similarly unable to transport proteins from the ER into the *cis*-Golgi, we used the assay system devised by Beckers et al. (1987), based on the acquisition of sensitivity to endoglycosaminidase D in cells deficient in N-acetyl glucosaminyl transferase. We found that in such a fibroblast cell line (LEC 1) infected with the temperature-sensitive mutant of the vesicular stomatitis virus (VSV) ts045, the newly synthesized-G protein remained resistant to endoglycosaminidase D at all times of chase at the permissive temperature, after permeabilization with SLO (data not shown). These observations were consistent with lack of export of newly synthesized proteins from the ER to the *cis*-Golgi in SLO-permeabilized cells. Permeabilization of the plasma membrane, thus, resulted in a cell with intact organelles, but no membrane traffic between organelles.

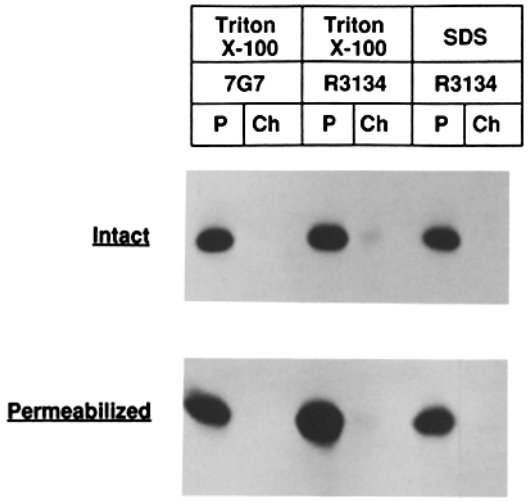

Figure 3. Loss of Tac-TCRα detected by immunoprecipitation with a monoclonal and a polyclonal antibody after solubilization with Triton X-100 or SDS. M1 cells expressing Tac-TCRα were pulse labeled for 15 min with [^{35}S]methionine. Cells were left intact or permeabilized and chased for 0 (*P*) or 3 h at 37°C (*Ch*). Proteins were solubilized in lysis buffer containing 0.5% Triton X-100, before immunoprecipitation with the mAb 7G7, or the polyclonal antibody R3134; or solubilized in lysis buffer containing 0.5% Triton X-100 and 1% SDS, before immunoprecipitation with R3134, as indicated in the figure.

Pulse-chase metabolic labeling experiments were performed to further analyze the intracellular fate of Tac and the chimeric proteins in intact and permeabilized cells. In intact cells, Tac was gradually processed upon transport into the Golgi system resulting in a shift in molecular mass from 45 to 55 kD (Fig. 2 *a*). The 45-kD form was sensitive to endo H, whereas the 55-kD form was resistant to endo H, as its carbohydrate side chains had been processed by Golgi enzymes (data not shown). A 40-kD soluble, endo H-resistant derivative of Tac, thought to arise by a proteolytic cleavage at the plasma membrane (Rubin et al., 1985*b*; Robb and Kutny, 1987), was slowly released into the medium (Fig. 2 *c*). In contrast, Tac-TCRα was rapidly degraded, with a half-time of ~10 min (Fig. 2 *b*). There was no appearance of a higher molecular weight, endo H-resistant form before degradation (Fig. 2 *b*), and no immunoprecipitable material was released into the medium (Fig. 2 *d*).

In permeabilized cells, normal Tac remained as a 45-kD precursor that was relatively stable ($t_{1/2}$ = 8.6 h) (Fig. 2 *e*). As permeabilization resulted in lack of transport into the Golgi system, neither the 55-kD mature form (Fig. 2 *e*) nor the secreted form was seen (Fig. 2 *g*). Thus, when normal Tac was not allowed to exit the ER, by virtue of permeabilization of the cells, it was not degraded. In contrast, Tac-TCRα was rapidly degraded in permeabilized cells (Fig. 2 *f*). During the chase period no processed form was detected (Fig. 2 *f*) and no immunoprecipitable Tac species was found in the cell's supernatant (Fig. 2 *h*).

On immunofluorescence microscopy of permeabilized cells, staining of Tac-TCRα was typical of that of the ER (Fig. 1 *d*). Treatment of permeabilized cells with 10 μg/ml cycloheximide revealed that staining of Tac-TCRα was rapidly lost and was localized exclusively to the ER at all time points (Fig. 1, *f* and *h*).

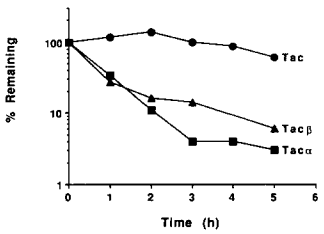

Figure 4. Kinetics of degradation of Tac-TCRα and Tac-TCRβ in permeabilized cells. Cells were metabolically labeled for 15 min at 37°C, permeabilized and chased for 0-5 h at 37°C. Detergent-solubilized Tac species were immunoprecipitated with 7G7 before analysis by SDS-PAGE. The amount of each protein relative to time 0 was determined by densitometric scanning of autoradiograms.

Loss of the chimeric protein by immunoprecipitation was observed using two mAbs, 7G7 (Fig. 3) and anti-Tac, as well as the rabbit polyclonal antibody, R3134, which recognizes both native and denatured forms of the Tac antigen (Fig. 3). Furthermore, disappearance of Tac-TCRα was observed when cell proteins were extracted with 1% SDS before immunoprecipitation with the polyclonal antibody R3134 (Fig. 3). We thus concluded that the disappearance of Tac-TCRα was not due to a conformational change causing loss of antibody recognition, or development of aggregates insoluble in Triton X-100, but rather due to protein degradation.

In seven independent experiments, the half-life of Tac-TCRα in permeabilized cells varied between 12 and 38 min (Fig. 2 *f* and Fig. 4), with a mean of 26 min and a standard error of 4 min. Another chimeric protein, Tac-TCRβ, comprised of the ectodomain of Tac and the transmembrane and cytoplasmic domains of TCRβ was also rapidly degraded in SLO-permeabilized fibroblasts ($t_{1/2}$ = 37 min) (Fig. 4). Thus, in permeabilized cells, Tac-TCRα and Tac-TCRβ were rapidly degraded, whereas normal Tac was only slowly turned over, just as in intact cells. The similar selectivity provided strong evidence that the processes involved in pre-Golgi degradation were preserved in the permeabilized cells.

Effect of Temperature on Degradation of Tac-TCRα in Permeabilized Cells

The pre-Golgi degradation of newly synthesized proteins in intact cells has been shown to exhibit a characteristic temperature dependence. Incubation of intact cells at temperatures of 18°C or lower strongly inhibits the degradation of the TCRα chain (Lippincott-Schwartz et al., 1988), the TCRβ and CD3δ chains (Wileman et al., 1990*b*), and the H2a subunit of the asialoglycoprotein receptor (Amara et al., 1989). Inhibition of degradation at these lower temperatures serves as a useful trade mark for this degradative pathway. To test whether the degradation of Tac-TCRα had a similar temperature dependence in permeabilized cells as in intact cells, the stable transfectants were labeled with [^{35}S]methionine, permeabilized with SLO, and chased for various periods over 2 h at different temperatures (37, 25, 20, 15, 10, and 4°C). As shown in Fig. 5, the degradation of Tac-TCRα was significantly inhibited at 25 and 20°C, and completely inhibited at 15°C or lower temperatures, similar to the previously described process in intact cells.

Involvement of Free Sulfhydryl Groups in Pre-Golgi Degradation

The presence of a thiol reducing agent in the assay buffer during the permeabilization and chase periods was a requirement for the occurrence of rapid degradation in permeabilized cells. Degradation was slow in the absence of a reducing agent (Fig. 6 *b*). Degradation was more rapid in the presence of 1 mM DTT (Fig. 6 *a*), than in the presence of 1 mM β-mercaptoethanol (not shown), presumably as the former is a more potent reducing agent. This observation predicted that a membrane-permeable thiol oxidizing reagent should be able to inhibit the same degradation system in intact cells. To test this prediction, we examined the effect on degradation of Tac-TCRα of a compound with these properties, "diamide" [Diazenedicarboxylic acid bis-(*N*,*N*-dimethylamide)]. Addition of 1 mM diamide to pulse-labeled intact

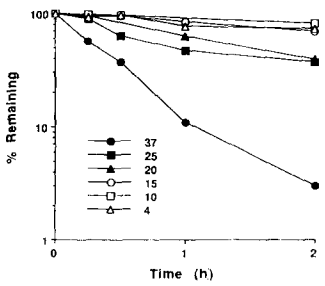

Figure 5. Inhibition of degradation of Tac-TCRα at low temperatures. Cells transfected with Tac-TCRα were metabolically labeled, permeabilized, and chased in permeabilization assay buffer at the indicated temperatures (in degrees Celsius). After immunoprecipitation with 7G7 and SDS-PAGE, the relative amount of protein with respect to time 0 was determined by densitometry.

cells resulted in a dramatic inhibition of Tac-TCRα degradation (Fig. 6 *d*). Inhibition of degradation by diamide was reversed when cells were washed and resuspended in fresh buffer containing 5 mM β-mercaptoethanol (2ME) (Fig. 6 *e*). Doses of diamide lower than 1 mM down to 0.25 mM partially inhibited degradation (data not shown). Diamide also inhibited pre-Golgi degradation of another sensitive substrate, CD4-TCRα (Bonifacino et al., 1990*b*), comprised of the ectodomain of CD4 and the transmembrane and cytoplasmic domains of TCRα (data not shown). These observations suggest that reduced sulfhydryl groups are required for rapid degradation both in intact and in permeabilized cells. Exactly how they participate in the degradative process remains to be established, although one interesting possibility is that the enzymes involved include cysteine proteases.

Rapid Degradation of Tac-TCRα Is Independent of Exogenously Added ATP or Cytosol

Having thus established that rapid degradation could be seen in permeabilized cells, with specificity and characteristics similar to those seen in intact cells, we then set out to determine what the biochemical requirements for this degradative process were. A number of transport events between membrane-bound organelles have been shown to be dependent on the presence of ATP and cytosolic factors (Beckers et al., 1987; Melançon et al., 1987; Doms et al., 1987; Baker et al., 1988; Block et al., 1988). We asked whether degradation of Tac-TCRα in Ml cells was ATP dependent. The permeabilization procedure itself resulted in loss of ATP, so that the ATP content of permeabilized cells was <5% that of intact cells. Depletion of ATP to levels of <0.2% by addition of the phosphodiesterase, apyrase (50 U/ml), in the presence of 0.4 mM $CaCl_2$, had no effect on the rate of degradation of Tac-TCRα in permeabilized cells (Fig. 7 *b*). Similarly, addition of 2 mg/ml hexokinase and 5 mM D-glucose to the assay buffer, which resulted in ATP levels of <0.5%, did not affect the degradation of Tac-TCRα in permeabilized cells (data not shown). Thus, depletion of ATP on top of that which resulted from the permeabilization procedure itself did not further slow the rate of degradation. We then asked whether addition of ATP would speed up the rate of degradation in permeabilized cells, perhaps to the somewhat faster rate seen in intact cells. An ATP-regenerating system, comprised of 1 mM ATP, 5 mM creatine phosphate, and 5 IU/ml creatine phosphokinase, added to the assay buffer during the chase period, however, did not alter the rate of degradation (Fig. 7 *c*). Similarly, addition of cytosol (0.6 mg/ml) alone

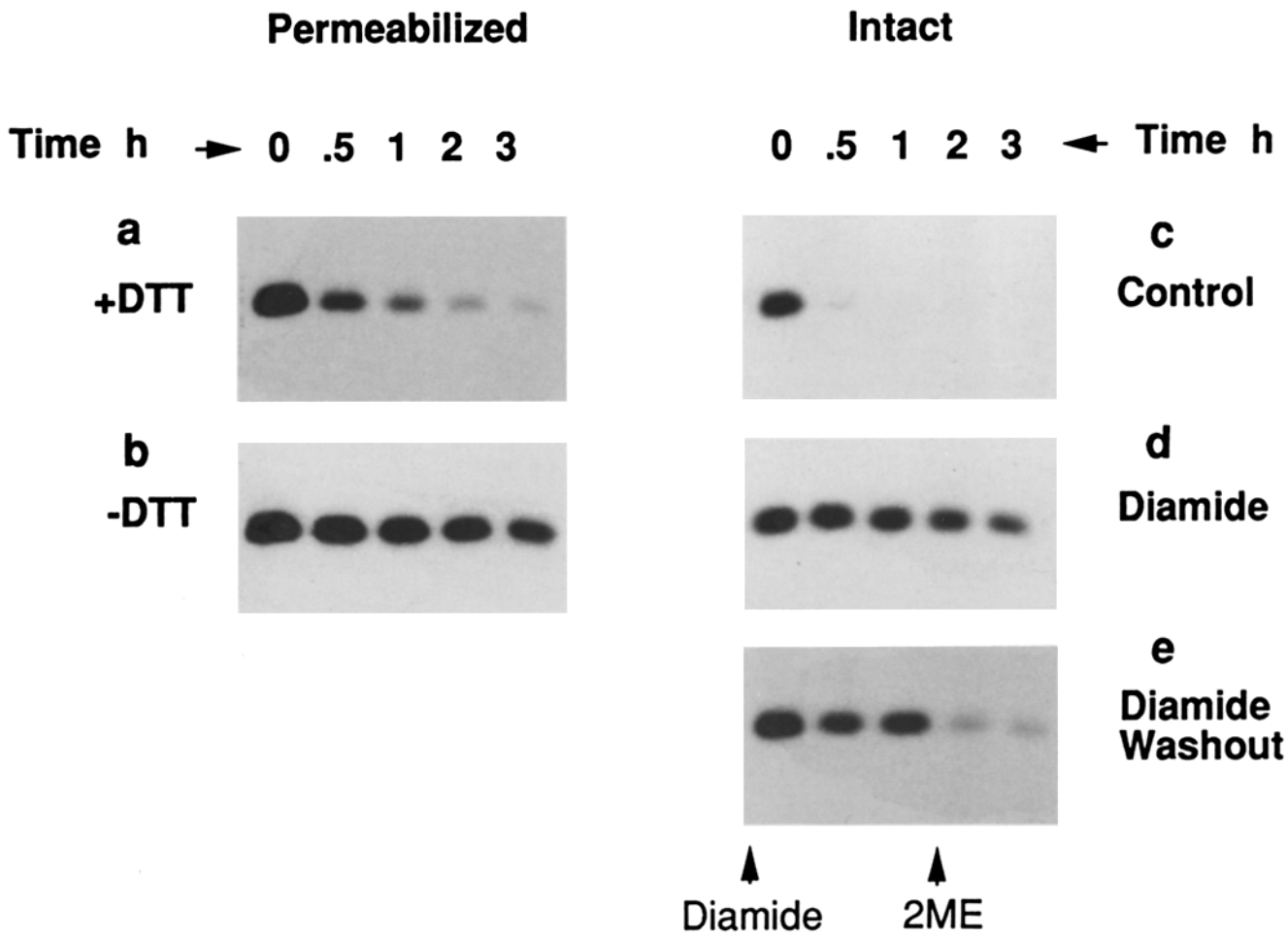

Figure 6. Requirement of free sulfhydryl groups in degradation of Tac-TCRα. Cells transfected with Tac-TCRα were metabolically labeled for 15 min at 37°C. Permeabilized cells were incubated in permeabilization assay buffer either (*a*) with 1 mM DTT or (*b*) without DTT for the chase periods indicated. Intact cells were incubated in (*c*) regular medium alone, (*d*) in the presence of 1 mM diamide for chase periods up to 3 h, or (*e*) in the presence of 1 mM diamide for 1 h and washed and resuspended in medium with 5 mM 2ME for a further 2 h. Proteins were solubilized and immunoprecipitated with 7G7 before analysis by SDS-PAGE.

(Fig. 7 *d*), or in combination with an ATP-regenerating system (Fig. 7 *e*) had no effect on the rate of degradation of Tac-TCRα. The lack of a requirement for exogenously added ATP and cytosol provided further evidence that degradation was independent of membrane traffic out of the ER.

Reagents That Block ER to Golgi Transport Do Not Inhibit Degradation of Tac-TCRα

Having established that factors necessary for ER to Golgi transport were not required for rapid ER degradation, we then asked whether reagents that block ER to Golgi transport would inhibit degradation. Evidence from studies of semi-intact cells has suggested that GTP plays an important role in the regulation of transport between the ER and the Golgi system (Beckers and Balch, 1988; Baker et al., 1988; Beckers et al., 1990). Incubation with the nonhydrolyzable guanine nucleotide analog, GTPγS, has been shown to inhibit transport in such a system (Beckers et al., 1990). In contrast to these effects on ER to Golgi transport, addition of 100 μM GTPγS did not slow, but rather increased the rate of degradation of Tac-TCRα (Fig. 8). Ca^{2+} ions have been shown to be essential for the transport of proteins between membrane-bound organelles, including ER to Golgi transport (Beckers and Balch, 1988; Beckers et al., 1990). In spite of the Ca^{2+} requirement for transport processes, however, addition of 5 mM EDTA, which chelates both Ca^{2+} and Mg^{2+}, or 5 mM EGTA, which chelates Ca^{2+} alone, did not slow degradation (Fig. 8). In keeping with a recent report that depletion of cellular calcium in intact cells accelerates pre-Golgi degradation (Wileman et al., 1991), EDTA and EGTA in our system reproducibly increased the rate of degradation of Tac-TCRα.

Rapid Protein Degradation in an Isolated Rough ER Fraction

Our data suggested that in permeabilized cells with intact organelles, but with no traffic between organelles, degradation of newly synthesized proteins proceeded as in intact cells, and thus, pointed to the ER as the site of proteolysis. We asked whether we could then demonstrate protein degradation in isolated fractions of rough ER. After metabolic labeling of cells for 15 min, a fraction containing rough ER was isolated by centrifugation of a postnuclear supernatant through a discontinuous sucrose gradient, as described by Bole et al., (1986). Rough ER was collected at the 2:1.3 M sucrose interface. Other organelles such as smooth ER, Golgi, lysosomes,

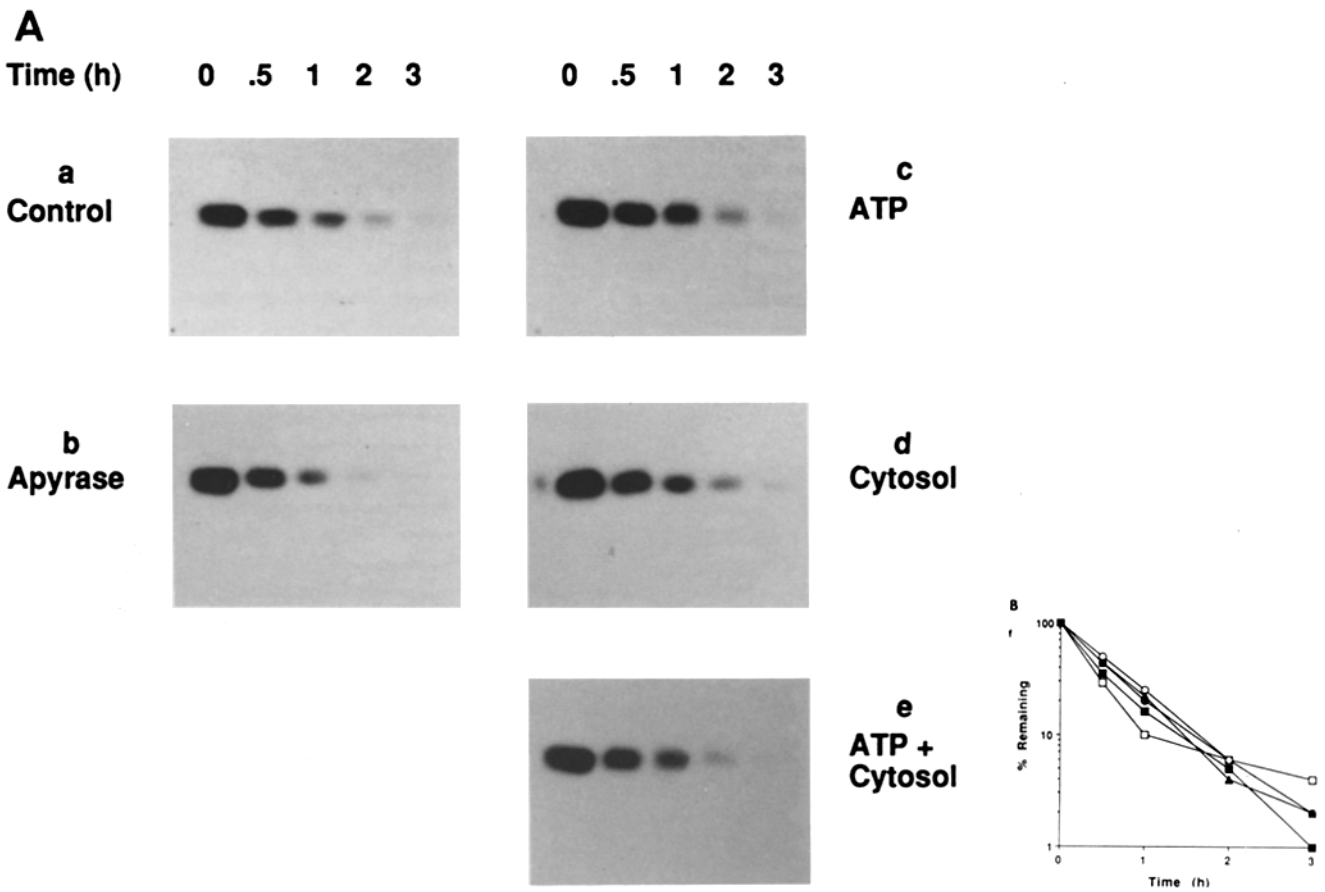

Figure 7. Degradation of Tac-TCRα is independent of ATP and cytosol. Cells transfected with Tac-TCRα were metabolically labeled and permeabilized. Cells were then incubated in the permeabilization assay buffer (*a*) alone, or with (*b*) apyrase, (*c*) an ATP-regenerating system, (*d*) cytosol, or (*e*) an ATP-regenerating system and cytosol. Proteins were immunoprecipitated with 7G7 before analysis by SDS-PAGE. (*f*) These autoradiograms were analyzed by densitometry, and the amount of each protein relative to time 0 was calculated. ●, control; □, apyrase; ○, ATP; ■, cytosol; ▲, ATP + cytosol.

and plasma membrane are known to band at lower densities in this type of gradient (Bole et al., 1986; Saraste et al., 1986). The freshly isolated rough ER fraction, containing ~24% of the total labeled Tac-TCRα in the postnuclear supernatant, was resuspended in assay buffer at 37°C, in the absence of added ATP or cytosol. Rapid degradation of Tac-TCRα was seen such that the protein had a half-life of ~11 min (Fig. 9). In contrast, normal Tac was relatively stable with a half-life of ~2 h (Fig. 9). Thus, a fraction enriched in rough ER was capable of degrading newly synthesized proteins with a specificity similar to that observed in intact and permeabilized cells.

Discussion

The identification of protein substrates that are especially sensitive to pre-Golgi degradation, such as some TCR chains and the chimeric proteins used here, has greatly facilitated the study of this pathway. The characteristic speed with which these transmembrane proteins are degraded, as well as the selectivity of this process, distinguish this type of degradation from other, perhaps less specific, degradative processes. By several measures, the degradation of newly synthesized proteins observed in cells permeabilized with SLO clearly corresponds to the same process originally described in intact cells. First, chimeric proteins containing TCR transmembrane sequences are rapidly degraded without production of detectable intermediates. Degradation of these proteins in permeabilized cells seems to occur at a slightly slower rate, as compared with intact cells ($t_{1/2}$ = 12–38 min vs. 10–20 min, respectively). This difference may be due to some loss of proteases or other factors involved in degradation during permeabilization. Second, as in intact cells, normal Tac is degraded at a much slower rate, thus confirming the selectivity of the process. Third, degradation in permeabilized cells shows a similar temperature dependence and is completely inhibited ~15°C. Fourth, degradation in both systems involves critical reduced sulfhydryl groups. To the extent to which they were studied, the efficiency and selectivity of degradation were also conserved in an isolated rough ER fraction.

The observation that permeabilized cells retain the ability to degrade newly synthesized proteins allowed examination of other characteristics of the degradative process. A fundamental question is where this degradation takes place. Even though most of the morphological and biochemical evidence was consistent with degradation occurring before transport into the Golgi system, the inability to specifically and completely interrupt transport between the ER and the Golgi system in intact cells made this interpretation uncertain. A complete block of transport between these two organelles can be achieved by permeabilization of cells

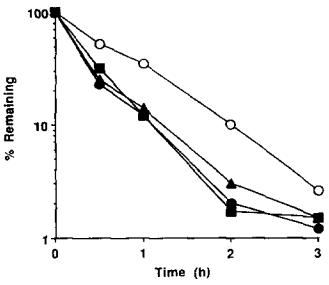

Figure 8. The rate of degradation of Tac-TCRα is not reduced by reagents which inhibit ER to Golgi transport. Cells were metabolically labeled, permeabilized, and chased in buffer alone, or in the presence of added 100 μM GTPγS, 5 mM EGTA, or 5 mM EDTA. Proteins were immunoprecipitated with 7G7 and analyzed by SDS-PAGE. The amount of protein remaining relative to time 0 was calculated from densitometric scanning of autoradiograms. ○, control; ●, EGTA; ■, EDTA; ▲, GTPγS.

(Beckers et al., 1987; Simons and Virta, 1987; Baker et al., 1988). Disruption of this transport pathway is due to loss of ATP and cytosol, as addition of these results in reconstitution of transport (Beckers et al., 1987; Baker et al., 1988). ATP and cytosol are probably required at several stages of the pathway, including the formation of intermediate carrier vesicles (Groesch et al., 1990; Beckers et al., 1990). Permeabilized cells have also been used to show that addition of Ca^{2+} chelators and nonhydrolyzable GTP analogues causes inhibition of transport between the ER and the Golgi system, suggesting a role for Ca^{2+} and G proteins in this process (Beckers and Balch, 1988; Beckers et al., 1990). These requirements are in fact common to other processes that involve traffic between membrane-bound organelles of the secretory system, such as intra-Golgi transport (Melançon et al., 1987), exocytosis (Salminen and Novick, 1987), and endocytosis and recycling from the cell surface (Podbilewicz and Mellman, 1990; Schmid and Carter, 1990). Our analysis of degradation of Tac-TCRα in permeabilized cells showed that this was independent of the presence or absence of ATP and cytosol in the medium and was not inhibited by Ca^{2+} chelators or a nonhydrolyzable GTP analogue, thus dissociating the occurrence of degradation from transport between the ER and the Golgi system.

The existence of exit routes for newly synthesized proteins other than through the Golgi system has not been definitively established, but even if they did occur, they would most likely involve membrane traffic with similar biochemical requirements. Hence, this pathway for the degradation of newly synthesized proteins appears to be confined to the ER system. This organellar system can be subdivided into several contiguous subcompartments including the rough ER, smooth ER, and nuclear envelope, each of which has a somewhat different composition and function (Palade, 1975). Some macromolecules, such as precursors of chondroitin sulfate proteoglycan and the associated link protein, are localized to specialized regions of the ER (Vertel et al., 1989). Our results do not rule out the possibility that degradation requires lateral movement from the site of synthesis in the rough ER to another ER subcompartment. However, the degradation in an isolated microsomal fraction of Tac-TCRα synthesized during a short pulse suggests that the ribosome-containing ER itself has the ability to effect protein breakdown.

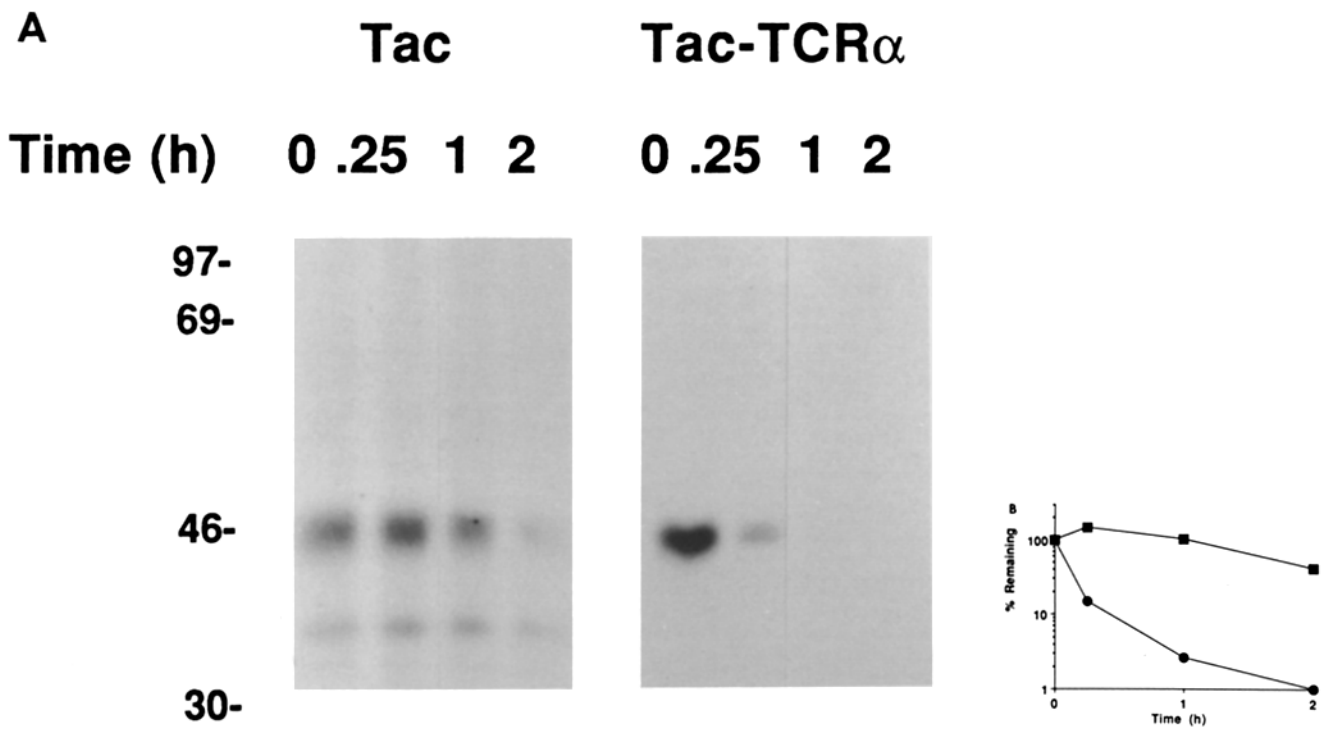

Figure 9. Degradation of Tac-TCRα in an isolated ER fraction. Cells transfected with Tac or Tac-TCRα were metabolically labeled for 15 min, and disrupted by nitrogen cavitation and dounce homogenization. Postnuclear supernatants were centrifuged through a discontinuous sucrose gradient. The rough ER fraction at the 1.3:2 M interface was resuspended in assay buffer for the indicated time periods. Proteins were immunoprecipitated with 7G7 and analyzed by SDS-PAGE. Densitometric scanning of these autoradiograms allowed measurement of the amount of each protein remaining relative to time 0, which is plotted in the graph shown. ■, Tac; ●, Tac-TCRα.

Other proteolytic processes thought to occur within the ER include the cleavage of signal peptides (Shelness et al., 1988), the removal of carboxyl terminal extensions upon addition of glycolipid membrane anchors (Cross, 1990), and the removal of accessory molecules such as the T-cell receptor associated protein (Antusch et al., 1990). The occurrence of these proteolytic processing events, together with the observation of rapid but selective protein breakdown within the ER, all indicate that the ER must contain proteases that perform these functions. Chymostatin-sensitive endopeptidase and zinc metalloendopeptidase activities have been noted to be associated with dog pancreas microsome membranes (Mumford et al., 1980). Except for signal peptidase, however, no other ER protease has been isolated and fully characterized to date. The ability to observe rapid degradation of certain protein substrates in isolated microsomes should serve as a starting point for the identification and purification of ER proteases involved in this process.

The finding that degradation of Tac-TCRα occurs in permeabilized cells even after severe ATP depletion is important not only because it argues against exit from the ER, but also because it suggests that the degradative process itself does not require ATP. Other degradative pathways, notably cytosolic protein breakdown, have been shown to involve ATP-dependent proteinases (Etlinger and Goldberg, 1977; Hough et al., 1987; Waxman et al., 1987). Degradation of both exogenous and endogenous proteins in lysosomes has also been found to require high levels of cellular ATP, although in this case ATP is used not for the degradative reaction itself, but for the delivery of proteins to lysosomes and for the maintenance of the optimal acidic environment necessary for lysosomal proteases. In spite of its independence from exogenously added ATP in permeabilized cells and isolated microsomes, however, combinations of metabolic poisons such as 2-deoxy-D glucose plus either dinitrophenol or sodium azide have been found to slow down the degradation of newly synthesized Tac-TCRα in intact cells by up to sixfold (F. J. Stafford, unpublished observations). These treatments are effective at lowering intracellular ATP levels (to $\sim$1%), but have a variety of other effects which might retard ER degradation in intact cells, such as triggering of a stress response, inhibition of protein synthesis, abnormal glycosylation, changes in redox state and others (Ananthan et al., 1986). Thus, partial inhibition by metabolic poisons in vivo should not necessarily be interpreted as indicating that pre-Golgi degradation is effected by ATP-dependent proteinases. A more isolated system such as permeabilized cells or microsomes, should be regarded as a better setting to define whether the degradative process itself needs ATP.

Another interesting characteristic revealed by the permeabilized cell system is the possible involvement of free sulfhydryls in degradation. The observation that reducing agents were required for degradation in permeabilized cells predicted that cell-permeable thiol oxidizing reagents would be effective inhibitors of pre-Golgi degradation in intact cells. That this was the case was demonstrated by the reversible inhibition effected by diamide. It is tempting to infer from this observation that at least some enzymes involved in ER degradation belong to the class of cysteine proteases. In fact, many intracellular proteinases that participate in cytosolic and lysosomal pathways of protein breakdown belong to this class (Bond and Butler, 1987). Cysteine proteases contain an essential sulfhydryl group at their active site that forms transient covalent complexes with their substrates (Polgar and Halasz, 1982). However, thiol reagents are not specific for active site sulfhydryls and could potentially affect the activity of other classes of proteases or other putative components of the degradative machinery. A more accurate assessment of the potential role of cysteine proteases in ER degradation will have to await the development of methods to deliver active site-specific cysteine reagents (i.e., peptide derivatives) into the membrane-bound compartment where degradation takes place.

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