Carboxy Terminally Truncated Forms of Ribophorin I Are Degraded in Pre-Golgi Compartments by a Calcium-dependent Process

Yung Shyeng Tsao, N. Erwin Ivessa, Milton Adesnik, David D. Sabatini, and Gert Kreibich

Department of Cell Biology, New York University School of Medicine, New York 10016

Abstract. Two COOH terminally truncated variants of ribophorin I (RI), a type I transmembrane glycoprotein of 583 amino acids that is segregated to the rough portions of the ER and is associated with the protein-translocating apparatus of this organelle, were expressed in permanent HeLa cell transformants. Both variants, one membrane anchored but lacking part of the cytoplasmic domain (RI_{467}) and the other consisting of the luminal 332 NH_2-terminal amino acids (RI_{332}), were retained intracellularly but, in contrast to the endogenous long lived, full length ribophorin I ($t_{1/2}$ = 25 h), were rapidly degraded ($t_{1/2}$ < 50 min) by a nonlysosomal mechanism. The absence of a measurable lag phase in the degradation of both truncated ribophorins indicates that their turnover begins in the ER itself. The degradation of RI_{467} was monophasic ($t_{1/2}$ = 50 min) but the rate of degradation of RI_{332} molecules increased about threefold ~50 min after their synthesis. Seveal pieces of evidence suggest that the increase in degradative rate is the consequence of the transport of RI_{332} molecules that are not degraded during the first phase to a second degradative compartment. Thus, when added immediately after labeling, ionophores that inhibit vesicular flow out of the ER, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and monensin, suppressed the second phase of degradation of RI_{332}. On the other hand, when CCCP was added after the second phase of degradation of RI_{332} was initiated, the degradation was unaffected. Moreover, in cells treated with brefeldin A the degradation of RI_{332} became monophasic, and took place with a half-life intermediate between those of the two normal phases. These results point to the existence of two subcellular compartments where abnormal ER proteins can be degraded. One is the ER itself and the second is a nonlysosomal pre-Golgi compartment to which ER proteins are transported by vesicular flow. A survey of the effects of a variety of other ionophores and protease inhibitors on the turnover of RI_{332} revealed that metalloproteases are involved in both phases of the turnover and that the maintenance of a high Ca^{2+} concentration is necessary for the degradation of the luminally truncated ribophorin.

LIKE any subcellular organelle, the ER contains proteins that permanently reside within it and function in its various biosynthetic and metabolic activities. However, it also contains proteins with other subcellular destinations that transit through the ER after they are inserted into its membrane or translocated into its lumen during the course of their synthesis on membrane-bound ribosomes (for review see Sabatini and Adesnik, 1989). Polypeptides synthesized in the ER not only undergo various co- and posttranslational modifications within the organelle, but are also folded and in some cases oligomerized to form multisubunit proteins within the ER itself (for review see Rose and Doms, 1988; Hurtley and Helenius, 1989), apparently assisted by resident proteins which prevent the egress of incompletely assembled products (Bole et al., 1986; deSilva et al., 1990; Siegel and Walter, 1989).

In recent years it has become apparent that many proteins that are normally transported from the ER to the Golgi apparatus, when improperly folded or incompletely assembled, undergo degradation within the ER by a proteolytic system that has not yet been fully characterized (for review see Klausner and Sitia, 1990). Many abnormal or unassembled polypeptide subunits are degraded very rapidly, with half-lives of 4–60 min (Lippincott-Schwartz et al., 1988; Stoller and Shields, 1989), whereas others, although apparently not leaving the ER, are much more stable (Bonifacino et al., 1989; Wileman et al., 1990*a*). For example, the α, β and δ-chains of the T cell receptor (TCR),[1] type I transmembrane polypeptides that remain in the ER when ex-

Yung Shyeng Tsao's present address is Schering-Plough Research, Union, NJ.

1. *Abbreviations used in this paper*: BFA, brefeldin A; BiP, heavy chain binding protein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; endo H, endoglycosidase H; RI_{332} and RI_{467}, truncated forms of ribophorin I containing its 332 and 467 NH_2-terminal amino acids, respectively; TCR, T cell receptor.

pressed in transfected cells in the absence of other subunits necessary for multimerization, undergo a rapid degradation, whereas the γ and ϵ subunits are stable, although they too are apparently retained in the ER (Lippincott-Schwartz et al., 1988; Chen et al., 1988; Bonifacino et al., 1989; Wileman et al., 1990*a*). In addition, a truncated variant of the α chain of the TCR that contains only the luminal domain, which is also retained in the ER, is much more stable than the intact polypeptide. In fact, it has been shown that in the case of the TCR α chain the rapid degradation of the native polypeptide depends on the presence of its specific transmembrane domain (Bonifacino et al., 1990). Transmembrane portions of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a permanent resident protein of the ER that traverses the membrane several times, have also been shown to play a critical role in determining the enhanced degradation rate of that protein under certain metabolic conditions (Jingami et al., 1987).

It is not clear to what extent the natural turnover of normal resident proteins of the ER involves the same degradative system that destroys defective and incompletely assembled polypeptide subunits of proteins that are normally exported from the ER. The turnover of some ER proteins, such as cytochrome P450 and its reductase, which accumulate at high levels after administration of some drugs and are degraded after removal of the inducer, has been shown to involve autophagy (Masaki et al., 1987), a process which requires lysosomal functions and in hepatocytes appears to be the major mechanism for overall cellular protein degradation (Gordon and Seglen, 1982). The ER degradation of abnormal and incompletely assembled polypeptides, however, occurs by a different pathway, since it is not affected by drugs that block lysosomal proteolysis (Lippincott-Schwartz et al., 1988; Stoller and Shields, 1989).

In this paper we studied the fate of two abnormal, COOH terminally truncated variants of ribophorin I (RI), a resident membrane glycoprotein of the ER that is segregated to the rough portions of this organelle (Kreibich et al., 1978*a*,*b*). This type I transmembrane protein is highly stable ($t_{1/2}$ = 25 h) (Rosenfeld et al., 1984) and is part of a supramolecular complex that includes other integral ER membrane proteins that are components of the polypeptide translocation apparatus and the ribosome binding sites present in the rough ER (Kreibich et al., 1978*a*,*b*; Marcantonio et al., 1984; Amar-Costesec et al., 1984; Yu et al., 1990). One of the truncated variants of ribophorin I we examined contained only a large portion of the luminal domain of the protein, while the other also included the transmembrane domain. Both of these polypeptides were found to be highly unstable and began to be degraded in the ER soon after their synthesis. Whereas the degradation of the membrane-anchored variant showed monophasic kinetics and appeared to be completed in the ER itself, the luminally truncated molecules were degraded with biphasic kinetics and we provide evidence that this reflects the transfer of a substantial fraction of them to a second pre-Golgi compartment, where an even more rapid degradation took place. Pharmacological studies indicated that degradation in both pre-Golgi compartments involves the participation of metalloproteases and that maintenance of a high Ca^{2+} concentration in the lumen of these compartments is essential for rapid degradation of the luminally truncated molecule.

Materials and Methods

Reagents

The pSP65 plasmid vector was purchased from Promega Biotech (Madison, WI). Polymerases, restriction endonucleases, and mungbean nuclease were from New England Biolabs (Beverly, MA) and Bethesda Research Laboratories (Gaithersberg, MD), nucleotides and endoglycosidase H (endo H) from Boehringer-Mannheim Biochemicals (Indianapolis, IN), and [^{35}S]-methionine (1,100 Ci/mmol) from new England Nuclear (Boston, MA). HBSS and MEM were from Whittaker Bioproducts Inc. (Walkersville, MD), methionine-free RPMI 1,640 from Mediatech (Washington, DC), and FBS from Hazleton Biologics, Inc. (Lenexa, KS). Cell culture-tested Geneticin (G 418), sodium butyrate, protein A-Sepharose CL-4B beads, protease inhibitors, and ionophores were obtained from Sigma Chemical Co. (St. Louis, MO), Trasylol (Aprotinin) from Mobery Chemical Corp. (New York), and En^{3}Hance from New England Nuclear Research Products (Boston, MA). When not specified, reagents were purchased from Fisher Scientific Co. (Pittsburgh, PA). Brefeldin A (BFA) was obtained as a gift from Dr. Richard Klausner (National Institute of Child Health and Human Development, Bethesda, MD). The anti-Bip antibody was kindly provided by Dr. D. Bole (Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI). The rabbit polyclonal antibodies against rat liver ribophorin I have been previously described (Marcantonio et al., 1984; Yu et al., 1990).

Plasmid Construction and Transfection of HeLa Cells with Truncated Rat Ribophorin I Genes

The rat ribophorin I cDNA (Harnik-Ort et al., 1987) was removed from the plasmid vector pGEM-1 by digestion with EcoRI and HindIII, enzymes which cleave at the 5′ end of the cDNA and within the 3′ untranslated region (residue 1964), respectively (Fig. 1 *B*), and subcloned into the plasmid vector pSP65. To produce the 3′ truncations in the cDNA, a chemically synthesized double-stranded oligonucleotide (Fig. 1 *A*) containing three termination codons and four restriction enzyme cleavage sites (NcoI at the 5′ end, HindIII at the 3′ end, and MluI and ClaI in between) was cloned between unique NcoI and HindIII sites within the 3′-noncoding region of the ribophorin I cDNA (Fig. 1 *B*) in the plasmid pSP65. When the ribophorin I cDNA was cut in its coding region with one of the restriction enzymes indicated on the map, it was possible to choose a cleavage site within the oligonucleotide so that cleavage at both sites, followed by mung bean–nuclease treatment and circularization, placed a termination codon in frame immediately after the ligation site.

By using this strategy it is possible to generate cDNAs coding for carboxy terminally truncated forms of ribophorin I that contain, in addition to the signal peptide (residues −1 to −22), various NH_2-terminal portions of the mature ribophorin I sequence. These constructs were cloned into the expression vector pSV2 tk neo, and permanent transformants of HeLa cells were obtained after selection for growth in the presence of 0.4% geneticin (Compton et al., 1989). In the work reported here, cloned HeLa cell transformants expressing the truncated ribophorin I polypeptides, RI_{332} or RI_{467}, were used. RI_{332} lacks the membrane-anchoring domain (residues 416–433) but contains a large portion of the luminal domain, including the *N*-glycosylation site at Asn_{275} used in mature ribophorin I, whereas RI_{467} only lacks the last 116 residues of the cytoplasmic domain (see Fig. 1, *D* and *E*). The two permanent transformant cell lines are referred to as HeLa-RI_{332} and HeLa-RI_{467}, respectively. To achieve higher levels of expression the cells were treated with 5 mM sodium butyrate (Kruh, 1982; Rizzolo et al., 1985) for 12 h and cultured for at least 1 wk before they were used for labeling experiments. This treatment was repeated when the level of expression of the transfected gene decreased significantly.

Cell Labeling, Immunoprecipitation, and Gel Electrophoresis

The transfected HeLa cells were plated in 35-mm dishes to a density of 5–8 $\times$ 10^5 cells per dish. After 24 h of incubation, they were rinsed twice with HBSS free of methionine, and incubated for 30 min in methionine-free RPMI 1640 to deplete the intracellular methionine pool. In pulse–chase experiments, cells were then labeled with [^{35}S]methionine in methionine-free RPMI 1640 (125 μCi/ml) for 5 min (unless noted otherwise) and then transferred to MEM containing 7% FBS and 5 mM unlabeled methionine. Chase periods were terminated by placing the dishes on ice.

Cells were washed with ice-cold PBS (150 mM NaCl, 50 mM Na-

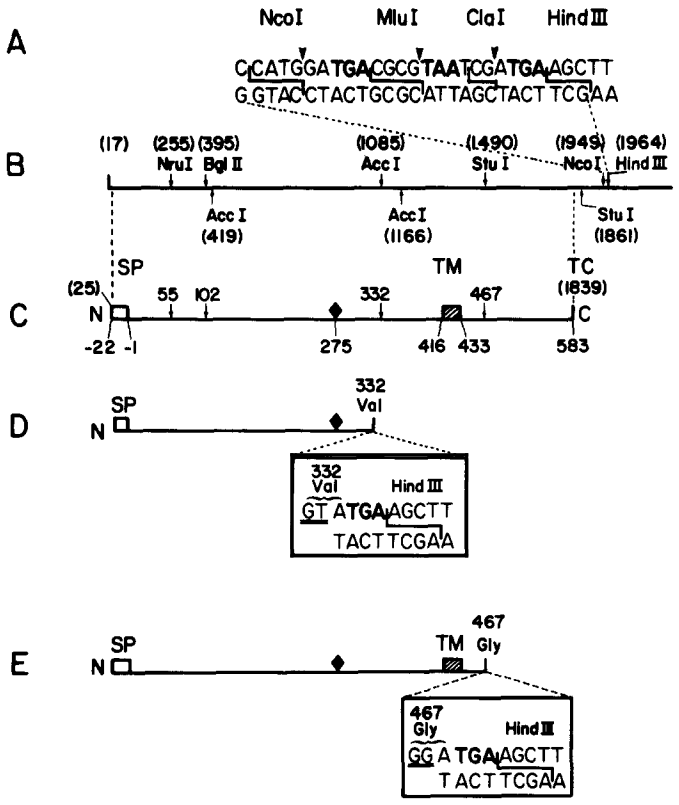

Figure 1. Construction of cDNA coding for truncated forms of ribophorin I. (*A*) Sequence of the synthetic oligonucleotide containing three termination codons (*bold face*) and four restriction cleavage sites (indicated on top) used to facilitate the construction of COOH-terminal deletion variants of ribophorin I. The cleavage sites are indicated and positions of blunt ends obtained after mung bean nuclease treatment are marked by arrowheads. (*B*) Partial restriction map of the ribophorin I cDNA. Only those enzymes which do not cut the pSP65 vector and were actually used to generate truncated forms of ribophorin I are included in the map. The dotted lines between *A* and *B* indicate the insertion of the oligonucleotide into the 3′-noncoding region of the ribophorin I cDNA. Throughout this figure, numbers in parenthesis refer to the position of nucleotides in the cDNA sequence, while numbers without parenthesis denote amino acid positions in the sequence of the primary translation product. (*C*) Schematic representation of the ribophorin I polypeptide. The open rectangle (□) denotes the signal peptide (*SP*; aa −22 to −1) and the hatched rectangle (▨) denotes the transmembrane domain (*TM*; aa 416–433). The glycosylated Asn residue is indicated by a diamond (♦) (aa 275). *TC* denotes the termination codon after aa 583. COOH-terminal deletions at the points indicated by arrows with the terminal amino acid residue numbers shown on top can be generated by cleavage with specific restriction enzymes. The numbers below the line that represents the polypeptide correspond to amino acid residues denoting specific features of the ribophorin I polypeptide. (*D*) Schematic representation of the truncated polypeptide containing the first 332 amino acids of intact ribophorin I ending at Val 332 (RI_{332}). The boxed nucleotide sequence illustrates the contributions of the ribophorin I cDNA (*double underlined*) and the oligonucleotide (*inset*) to the mutated cDNA that encodes the truncated product. (*E*) Schematic representation of the truncated polypeptide containing the first 467 amino acids of intact ribophorin I ending at Gly 467 (RI_{467}). (EMBL/GenBank/DDBJ under accession number X05300: rat rib 1.)

phosphate, pH 7.4) and lysates prepared in SDS-containing buffer (1 mg/ml immunoglobulin-free BSA, 95 mM NaCl, 3 mM EDTA, 2% SDS, 25 mM Tris-HCl, pH 7.4, 1 mM DTT, 0.5 μg/ml leupeptin, 1 μg/ml leu-leu-leu, and 20 U/ml traysol) by scraping off the cells in lysis buffer with the aid of a rubber policeman. Lysates were transferred to Eppendorf tubes, sonicated twice for 15 s using a microtip (Heat Systems-Ultrasonics, Inc., Plainview, NY), boiled for 2 min and finally diluted fourfold with a dilution buffer that had the same composition as lysis buffer, but contained 0.2% SDS and 1.25% Triton X-100. All subsequent steps were carried out at 4°C. The diluted cell extracts were centrifuged for 15 min at 15,000 *g* in a centrifuge (model 5412; Eppendorf Inc., Fremont, CA) to remove unsolubilized material and were then incubated with protein A-Sepharose CL-4B beads for 30 min and recentrifuged for 2 min in the Eppendorf centrifuge to remove material that binds nonspecifically to the beads. The supernatants were incubated overnight with the polyclonal anti-ribophorin I serum in a 1:500 dilution in the presence of protein A-Sepharose CL-4B beads. The beads were washed three times with dilution buffer and twice with PBS. Immunoprecipitates were dissolved in sample buffer (20% glycerol, 5% SDS; 10 mM Tris-HCl, pH 7.4), boiled for 5 min, and analyzed by SDS-PAGE (10% gels) (Laemmli, 1970). The gels were prepared for fluorography with EN^{3}HANCE, dried and exposed at −70°C using film (X-Omat AR; Eastman Kodak and Co., Rochester, NY). When necessary, immunoprecipitates were treated with endo H, as described (Rosenfeld et al., 1984).

To quantitate levels of labeled proteins, fluorograms were scanned using an optical scanning densitometer (model GS300; Hoefer Scientific Instruments, San Francisco, CA). The intensities of the bands corresponding to the truncated ribophorins were normalized with respect to the intensity of the endogenous intact ribophorin I band in the same lane. When pulse periods of only 5 min were used, the highest intensity of labeling of the truncated ribophorin relative to that of the endogenous HeLa cell intact ribophorin I occurred at 10 min of chase. Therefore, the relative intensities at different chase times were expressed as percentages of the 10-min value. First order rates of degradation were obtained by linear regression analysis of semi-log plots using the Minitab™ program (release 7.2; Minitab Project, University Park, PA). Since the decay curve for RI_{332} suggested a biphasic degradation, "coefficients of determination" were calculated for all possible second phases to determine the optimal break point between the two phases.

Sequential Immunoprecipitation with Anti-BiP and Anti-Ribophorin I Antibodies

HeLa-RI_{332} cells (5×10^5 cells per 60-mm tissue culture dish) were grown in MEM, containing 5% dialyzed FBS, 400 μg neomycin/ml, and 25 μCi [^{35}S]methionine/ml, for 48 h with one medium replacement halfway through the incubation. After a chase incubation for 3.5 h, the cells were starved for methionine (30 min in serum-free MEM without methionine) and labeled again, this time for 10 min, with 125 μCi [^{35}S]methionine/ml in serum-free MEM. The cells were then lysed in a nondenaturing buffer containing 1% NP-40, 0.05% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM D-glucose, 5 U/ml hexokinase (type VI, from bakers yeast, obtained from Sigma Chemical Co.), and protease inhibitors (see also Dorner et al., 1987). Two equal aliquots of the lysate (12% of the total volume) were used for immunoprecipitation with a mouse monoclonal anti-BiP antibody (4.8 μl). Immunocomplexes recovered using protein A-Sepharose CL-4B beads were washed successively with lysis buffers containing 1% NP-40, 0.5% NP-40, and then 0.1% NP-40 without SDS, before a final wash with PBS. The immunoprecipitate obtained from one aliquot was analyzed directly by SDS-PAGE, whereas the other was eluted from the beads by boiling for 3 min in 2% SDS lysis buffer and subjected to a second immunopre-

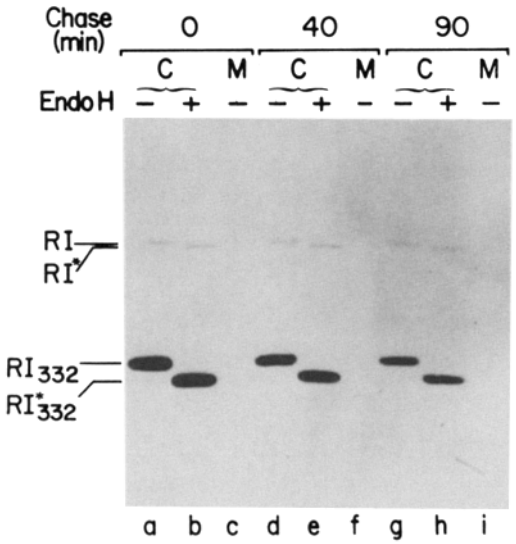

Figure 2. A truncated form of ribophorin I (RI_{332}) synthesized in HeLa cell transformants is not secreted. HeLa-RI_{332} cells were labeled with [^{35}S]methionine for 90 min and chased for 0 (lanes *a*, *b*, and *c*), 40 (lanes *d*, *e*, and *f*), or 90 (lanes *g*, *h*, and *i*) min. The media (*M*) were recovered and the cells (*C*) were lysed in an SDS-containing buffer. The cell lysates were divided into two equal aliquots and media (*M*; lanes *c*, *f*, and *i*) and cell lysates (*C*; lanes *a*, *b*, *d*, *e*, *g*, and *h*) were immunoprecipitated with a polyclonal anti-ribophorin I antibody. The immunoprecipitates obtained from one aliquot of each cell lysate were subsequently digested with endo H (lanes *b*, *e*, and *h*). All samples were analyzed on SDS polyacrylamide (10%) gels and fluorographs (12 h) were obtained.

cipitation using anti-ribophorin I antibodies. To do this, the beads were removed by sedimentation and 3 vol of dilution buffer containing 0.2% SDS and 1.25% Triton X-100 (see above) were added to the supernatant before addition of anti-ribophorin I antibody and fresh protein A-Sepharose beads. In parallel to this, a third equal aliquot of the lysate was used for immunoprecipitation under the usual denaturing conditions with anti-ribophorin I antibodies, so that the amount of RI_{332} recovered after sequential immunoprecipitation with anti-BiP and anti-RI_{332} antibodies could be compared with the total amount of RI_{332} in the sample.

Results

Rapid Turnover of Truncated Ribophorins: Biphasic Degradation of the Anchorless Variant

Intact rat ribophorin I (583 amino acids) consists of a luminal domain of 415 residues that has a single N-linked, high-mannose oligosaccharide chain at Asn_{275}, a transmembrane segment extending from residues 416 to 433, and a cytoplasmic domain 150 amino acids in length (Harnik-Ort et al., 1987). We first examined the fate of a truncated form of ribophorin I (RI_{332}) that lacks the cytoplasmic and transmembrane domains, as well as 84 amino acids of the luminal domain, in permanent transformants of HeLa cells expressing this protein. Immediately after labeling with [^{35}S]methionine for 90 min (Fig. 2, lanes *a–c*), or after chase periods of 40 and 90 min (Fig. 2, lanes *d–f* and *g–i*), an intensely labeled polypeptide of the size expected for the truncated ribophorin (M_r = 38 kD) was detected by immunoprecipitation in cell lysates (Fig. 2, lanes *a*, *d*, and *g*) but at no time was it found in the culture medium (Fig. 2, lanes *c*, *f*, and *i*). Like the endogenous intact glycoprotein (Rosenfeld et al., 1984), the truncated ribophorin I remained endo H sensitive throughout the labeling and chase periods (Fig. 2, lanes *b*, *e*, and *h*). This could be taken as evidence that this polypeptide does not reach the medial region of the Golgi apparatus. In separate work,[2] we have demonstrated that this is, in fact, the case. The failure of ribophorin I and its truncated derivative to become endo H resistant appears, however, to reflect incapacity of these polypeptides to serve as substrates for the Golgi enzymes that modify core oligosaccharide chains.[2]

The time-dependent changes in the intensities of the ribophorin I and RI_{332} bands in Fig. 2 suggest that the intact and anchorless ribophorin I molecules synthesized in the same transformants have markedly different stabilities. This is strikingly demonstrated in Fig. 3 *A* in which the relative amounts of labeled RI_{332} and endogenous ribophorin I can be compared after different chase periods extending up to 24 h. Although much more heavily labeled than the endogenous protein at the end of the pulse period, the truncated ribophorin was no longer detectable after 8 h of chase, while at this time the intensity of the band corresponding to ribophorin I had undergone little change.

To determine whether the rapid turnover of RI_{332} was solely a consequence of its lacking the membrane anchor, the kinetics of degradation of RI_{467}, a polypeptide that in addition to the entire luminal domain of ribophorin I contains the transmembrane segment and only 34 amino acids of the cytoplasmic domain, was examined. It was found that RI_{467}, which is also synthesized in large amounts in the correspond-

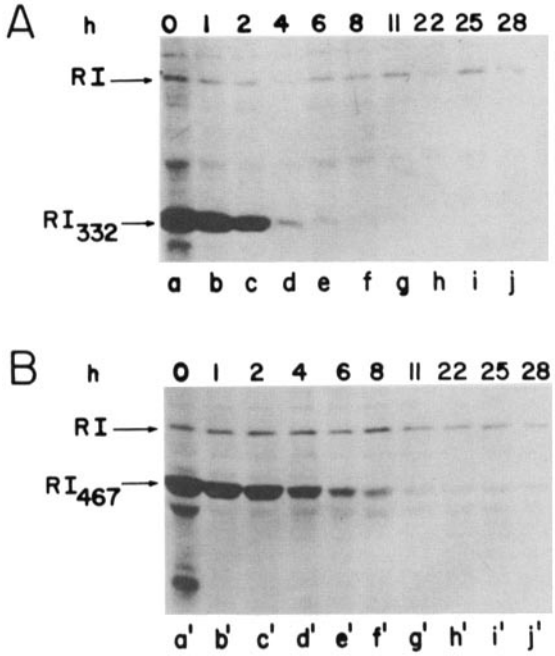

Figure 3. Two truncated forms of ribophorin I, one that is still membrane anchored and another that contains only part of the luminal domain, are rapidly degraded. HeLa-RI_{332} (*A*) and HeLa-RI_{467} cells (*B*) were preincubated in a methionine-free medium for 30 min, labeled with [^{35}S]methionine for 30 min, and chased for up to 28 h. Immunoprecipitates obtained from cell lysates were analyzed by SDS-PAGE followed by fluorography.

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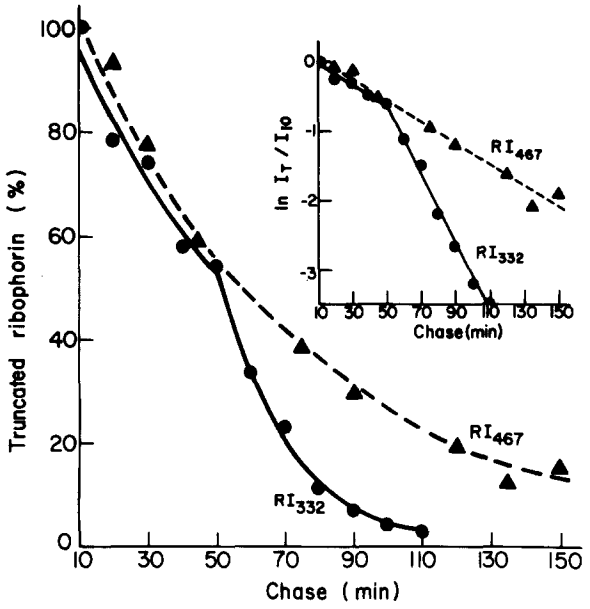

Figure 4. The two truncated forms of ribophorin I are rapidly degraded but with a different kinetics. HeLa-RI_{332} (●) or HeLa-RI_{467} cells (▲), respectively, were preincubated in a methionine-free medium for 30 min and pulse labeled with [^{35}S]methionine for 5 min. Immunoprecipitates of cell lysates obtained after chase periods of up to 150 min were analyzed by SDS-PAGE and the resulting fluorograms were quantitatively evaluated as described in Materials and Methods. In the inset, a semi-log plot of the same data shows the first order degradation kinetics for the two proteins.

ing HeLa cell transformants, also undergoes rapid degradation, albeit at a significantly slower rate than the anchorless ribophorin (Fig. 3 *B*). A quantitative kinetic analysis of the degradation of the two truncated ribophorins carried out in cell transformants that were labeled for 5 min and chased for closely spaced intervals extending up to 150 min (Fig. 4), showed that whereas the decay of RI_{467} follows simple first order kinetics with a half-life of ~50 min, the degradation of the anchorless protein is biphasic.

The biphasic character of the degradation of RI_{332} was clearly demonstrated by an analysis of decay data from six independent experiments (Fig. 5) which gave half-lives of 59 ± 12 and 18 ± 6 min for the first and second degradative phases, respectively, with the first phase lasting for 49 ± 6 min. A two-sample *t* test showed that the values of the two half-lives of RI_{332} are significantly different at a level of $P = 0.001$. It is perhaps fortuitous that the half-life of the first phase of degradation of RI_{332} is similar to that of RI_{467} ($t_{1/2}$ = 47 min).

Since both truncated proteins began to be degraded immediately after their synthesis, it can be presumed that the degradation process begins in the ER itself. The monophasic character of the turnover of RI_{467} indeed suggests that the degradation of this polypeptide is completed in the ER. On the other hand, the existence of a second accelerated phase of degradation for RI_{332} is compatible with the notion (tested experimentally below) that molecules of this type that have not been degraded in the ER after 50 min are transferred to a second compartment where they are degraded more rapidly.

We considered, however, the alternative possibility that the two phases of degradation of RI_{332} reflect a transition in the folding or assembly state of the protein. It is known (Hurtley and Helenius, 1989) that many improperly folded proteins synthesized in the ER become associated with BiP, a resident ER protein that may also assist in the assembly of some multisubunit proteins. It was therefore conceivable that the initial slow phase of RI_{332} degradation reflects its association with BiP and that the degradative rate increased when this association was terminated. This possibility was examined using anti-BiP antibodies to precipitate this protein, and any polypeptide associated with it, from cell extracts treated with NP-40, a nondenaturing detergent that preserves BiP-containing complexes (Machamer et al., 1990). For these experiments, the transfected cells were incubated with [^{35}S]methionine for 48 h to label BiP, which is a relatively stable protein, and after a chase period of 4 h, incubated again with [^{35}S]methionine for 10 min to label the newly synthesized RI_{332} molecules. The amount of newly synthesized RI_{332} polypeptides present in the cells was assessed in a parallel sample by direct immunoprecipitation with anti-ribophorin I antibodies under denaturing conditions. It was found (Fig. 6, lane *a*) that no labeled truncated RI_{332} molecules were recovered in the immunoprecipitates obtained with anti-BiP antibodies under nondenaturing conditions (Fig. 6, compare lane *a* with *c*), although large amounts of BiP itself were precipitated. Thus, solubilization of the anti-BiP immunoprecipitate with SDS followed by precipitation with anti-ribophorin I did not yield either intact or truncated ribophorin I (lane *b*). It can therefore be concluded that an association with BiP is not responsible for the initial slower rate of degradation of RI_{332} molecules.

It is noteworthy that in all the pulse-chase experiments that were carried out (see Figs. 2 and 3) the apparent molecular weight of RI_{332} remained constant throughout both phases of degradation. Since we have shown that in BFA-treated cells, the apparent molecular weight of the truncated ribo-

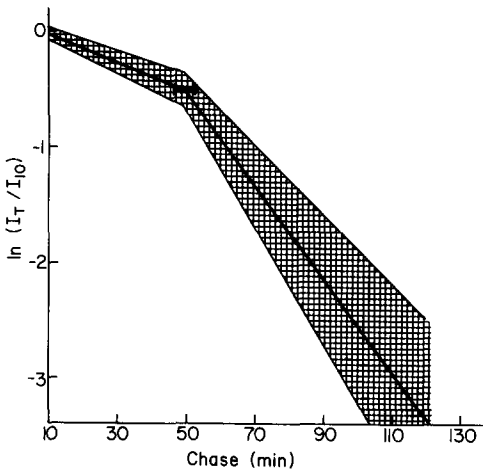

Figure 5. A truncated form of ribophorin I consisting of a large portion of the luminal domain is degraded with a biphasic kinetics. Data from six independent experiments were used to calculate the mean values for the two decay rates (k_1 = 0.012 ± 0.002 min^{-1}; k_2 = 0.041 ± 0.010 min^{-1}) and for the transition time between the two phases (t_{TR} = 49 ± 6 min). A biphasic decay curve for RI_{332} was plotted using these mean parameters. The shaded area indicates one standard deviation of the rates and the error bar the standard deviation of the transition time.

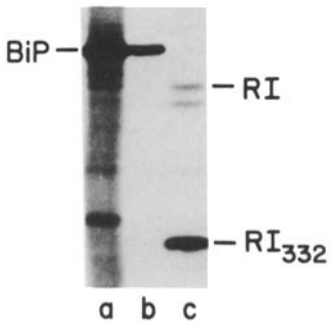

Figure 6. Newly synthesized RI_{332} does not associate with BiP. HeLa-RI_{332} cells were first labeled with [^{35}S]methionine for 48 h and, after a chase of 4 h labeled again with the same precursor for an additional 10 min. Cell lysates were prepared in a nondenaturing buffer containing 1% NP-40 and 0.05% SDS, and two aliquots were used for immunoprecipitation with anti-BiP antibodies. The immunoprecipitates were eluted from the protein A–Sepharose beads with SDS and one sample (*a*) was directly loaded onto the gel, whereas the second (*b*) was submitted to a second cycle of immunoprecipitation using anti-ribophorin I (lane *b*), as described in Materials and Methods. A third aliquot of the lysate (*c*) was denatured in SDS and immunoprecipitated with anti-ribophorin I under standard conditions (see Materials and Methods). Samples were analyzed on an 8% SDS-polyacrylamide gel and the fluorograph was exposed for 4 d. The BiP recovered in lane *b*, in which the second immunoprecipitation was carried out with anti-ribophorin I antibodies, is probably brought down by renatured anti-BiP antibody present in the immunoprecipitate recovered in the first cycle of immunoprecipitation.

phorin increases as a result of its *O*-glycosylation by Golgi enzymes relocated to the ER, it may be concluded that transfer of RI_{332} molecules from the ER to the second compartment does not involve their passage through the Golgi apparatus.[2] Since RI_{332} exhibits both phases of intracellular degradation, transformants expressing this protein were used to further characterize the degradative processes.

Inhibitors of Lysosomal Function or Autophagy Do Not Prevent the Degradation of Truncated Ribophorins

Direct evidence that lysosomal enzymes are not involved in the rapid turnover of RI_{332} was obtained by examining the effect on this process of agents that inhibit lysosomal function (Seglen, 1983), such as the lysosomotrophic drugs ammonium chloride and chloroquine, and various lysosomal protease inhibitors (Fig. 7). None of these agents, nor other drugs, such as colchicine and methyladenine, which inhibit autophagy, or cycloheximide (Seglen, 1983), which inhibits lysosomal function by an unknown mechanism, affected the turnover of RI_{332}, as indicated by the amount of radioactive protein remaining in the cell after a 90-min chase period in the presence or the absence of each agent. In this respect, the degradation of the truncated ribophorin resembles that of the α chain of the T cell antigen receptor complex, which takes place in the ER and is not inhibited by agents that block lysosomal function (Lippincott-Schwartz et al., 1988).

Calcium Ionophores Completely Prevent the Degradation of RI_{332}, while Monensin and CCCP Prevent the Occurrence of the Second Phase, Probably by Blocking Transport of the Truncated Ribophorin to the Second Degradative Site

The effect of a variety of cation-specific ionophores on the degradation of RI_{332} was first examined by measuring the fraction of RI_{332} that remained after a 90-min chase period

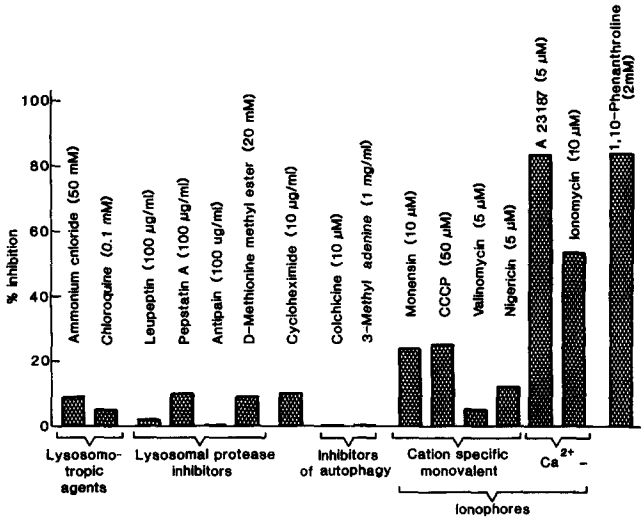

Figure 7. Degradation of RI_{332} does not occur in lysosomes. HeLa-RI_{332} cultures were preincubated for 2 h with the drugs indicated, except for cycloheximide, the cation-specific ionophores, and 1,10-phenanthroline, which were added at the time of the chase. The final concentration of each drug is indicated above the respective bar. Cells were pulse-labeled for 5 min with [^{35}S]methionine and chased for 90 min in the presence of the inhibitors. Immunoprecipitates obtained from cell lysates were analyzed by SDS-PAGE followed by fluorography. The decrease in the normalized intensities of the bands corresponding to RI_{332} were compared with that obtained from an untreated culture and percentages of inhibition were calculated.

in the presence of each agent (Fig. 7). The calcium ionophores A23187 and ionomycin almost completely blocked the turnover of RI_{332}, suggesting that appropriate concentrations of intracellular calcium are required for the degradation. CCCP, a proton ionophore that inhibits oxidative phosphorylation (Heytler, 1963), and monensin, a carboxylic ionophore that preferentially facilitates the transport of Na^+ over K^+ across membranes (Pressman and Fahim, 1982), also markedly reduced the degradation of the anchorless ribophorin in the 90-min chase period examined, but to a significantly lesser extent than the calcium ionophores. Since monensin and CCCP are known to inhibit vesicular transport between membranous organelles (Tartakoff et al., 1977; Tartakoff, 1983*a*,*b*), it was conceivable that they exerted their effect by blocking transport of RI_{332} from the ER to the site where the second phase of degradation takes place. Indeed, kinetic studies showed that in the presence of monensin (Fig. 8 *A*) or CCCP (Fig. 8 *B*) the degradation of RI_{332} was monophasic, proceeding to completion with half-lives similar to that of the first degradative phase observed in untreated cells. The prolongation of the first phase in cells treated with the ionophores establishes that it does not simply represent a lag period before true degradation begins.

To determine if these ionophores acted by preventing the transition from one phase to another, CCCP was added after 60 min of chase, a time when the second degradative phase had already begun. We found that in this case, (Fig. 8 *B*) CCCP had no effect on the degradation, which proceeded to completion at the same rate as in untreated cells. This indicates that the ionophore does not slow down the second phase of degradation, but rather, prevents the transition be-

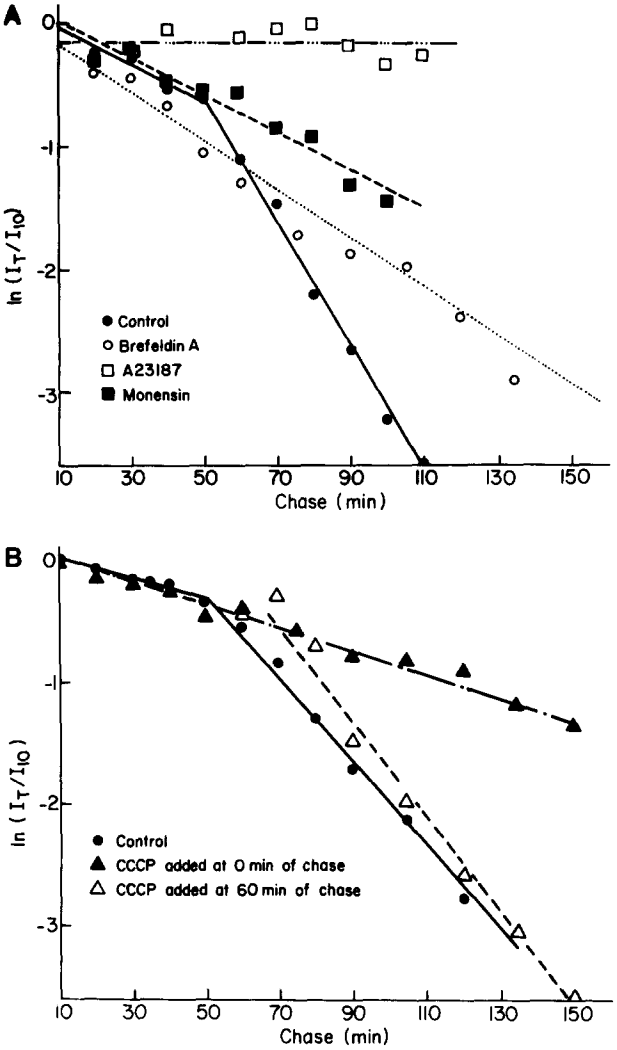

Figure 8. Pharmacological analysis of the degradation of RI_{332}: Complete inhibition by Ca^{2+} ionophores, prevention of the occurrence of the second phase of degradation by monensin and CCCP, and monophasic degradation at an intermediate rate in the presence of BFA. HeLa-RI_{332} cells were pulse labeled for 5 min with [^{35}S]-methionine and chased for up to 2 h in the absence (●) or in the presence of various drugs affecting the turnover of RI_{332}. The Ca^{2+} ionophore A23187 (5 μM; □) was added together with the chase medium, while cells treated with monensin (10 μM; ■) or BFA (5 μg/ml; ○) were, in addition, preincubated with these drugs for 2 h or 30 min, respectively. (*B*) CCCP was either added together with the chase medium (▲) or after 60 min of chase (△). The control (●) in this experiment, done in parallel with the CCCP-treated samples shows somewhat slower decay rates than average. Samples obtained at different chase periods were processed for immunoprecipitation, SDS-PAGE, and fluorography, and the data analyzed as described in Materials and Methods.

tween the two phases, as expected if it blocked transfer of RI_{332} between two degradative sites. A similar experiment cannot be carried out with monensin since there is a significant delay after its addition before this drug exerts its physiological effects on the cells.

In contrast to the effect of CCCP, the calcium ionophore A23187 completely blocked the degradation process when it was added at the beginning of the chase period (Fig. 8 *A*),

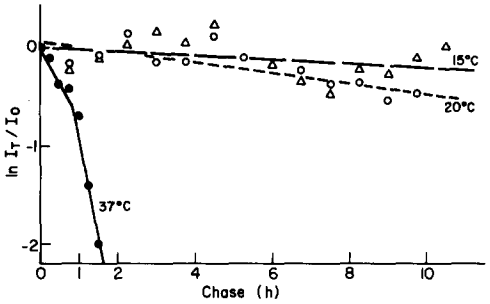

Figure 9. Degradation of RI_{332} is blocked in cells incubated at 15 or 20°C. HeLa-RI_{332} cells were pulse labeled with [^{35}S]methionine for 5 min and chased at 37°C (●), 20°C (○), or 15°C (△) for up to 10.5 h. Immunoprecipitates obtained from cell lysates were analyzed by SDS-PAGE followed by fluorography. The degradation rates were determined as described in Materials and Methods.

or after the second phase of degradation had begun (results not shown). This inhibitory effect of A23187 was also observed when it was added to the medium together with EGTA (not shown), indicating that the ionophore acted by depleting Ca^{2+} pools within the degradative compartments. Other ionophores specific for potassium ions, such as the neutral valinomycin and the carboxylic nigericin (Pressman and Fahim, 1982), had no significant effect on the degradation of RI_{332} (Fig. 7).

Further support for the notion that the first phase of degradation takes place in the ER, whereas the second takes place in a physically distinct compartment to which the molecules are transported by vesicular flow, was provided by experiments in which the cells were treated with BFA, a drug which leads to the back flow of Golgi constituents into the ER (Lippincott-Schwartz et al., 1989; Doms et al., 1989; Ulmer and Palade, 1989). In this case, the turnover of RI_{332} also became monophasic (Fig. 8 *A*) but degradation proceeded at a rate intermediate between those of the two phases observed in control cells ($t_{1/2}$ = 34 min). This suggests that BFA leads to the intermixing of components of the second site of degradation with those of the ER.

Degradation of RI_{332} Does Not Take Place at 15 or 20°C

Since it has been previously shown that vesicular transport from the ER to Golgi cisternae proceeds in cells incubated at 20°C but is halted when the temperature is reduced to 15°C (Saraste and Kuismanen, 1984; Saraste et al., 1986; Tartakoff, 1986; Tooze et al., 1988), we examined the effect of incubation at these low temperatures on the kinetics of degradation of RI_{332}. We found, however, that at both temperatures turnover of the truncated ribophorin was almost completely suppressed (Fig. 9). Although the role of vesicular flow between the two putative sites of degradation could not be assessed from these experiments, the pronounced temperature effect ($t_{1/2}$ = 14 h at 20°C and $t_{1/2}$ = 32 h at 15°C) on the degradation rate suggests that this process involves membrane-bound enzyme(s) whose activity depends markedly on the fluid state of the lipid bilayer (De Pierre and Dallner, 1975; Mitropoulos et al., 1980) and does not take place wholly in a homogeneous soluble phase (Burdette and Quinn, 1986).

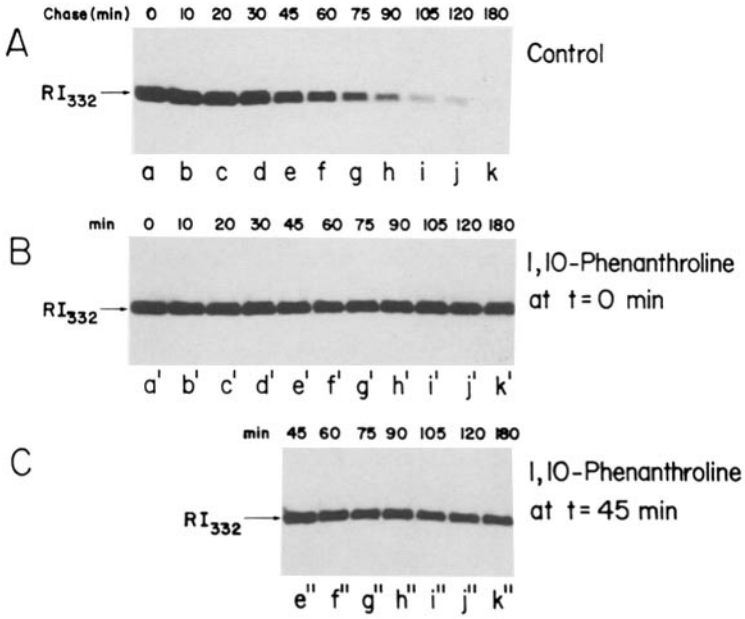

Figure 10. Both phases of degradation of RI_{332} involve the function of metalloproteases. After pulse labeling for 5 min with [^{35}S]methionine, HeLa-RI_{332} cell cultures were incubated under chase conditions for up to 3 h with no additions (*A*), with 1,10-phenanthroline (2 mM) added immediately after the pulse (*B*), or after 45 min of chase (*C*). Immunoprecipitation and data analysis were carried out as described in Materials and Methods.

1,10-Phenanthroline, an Inhibitor of Metalloproteases, Prevents Degradation of RI_{332}

The turnover of RI_{332} was prevented when 2 mM 1,10-phenanthroline, a chelator of divalent cations (such as Zn^{2+} and Fe^{2+}), which inhibits metalloproteases (Chlebowski and Coleman, 1976), was added to the cells immediately after the pulse (Fig. 10 *B*). Degradation was also halted when the inhibitor was added after 45 min of chase (Fig. 10 *C*). Similar results (not shown) were obtained when the cells were incubated with 3 mM of the dipeptide cbz-glycylphenylalanylamide, another metalloprotease inhibitor (Chlebowski and Coleman, 1976). These findings indicate that metalloproteases are involved in both degradative phases of the truncated ribophorin.

Discussion

The experiments just presented show that, in contrast to the intact ribophorin I, which is a highly stable protein ($t_{1/2}$ = 25 h) (Rosenfeld et al., 1984), two truncated ribophorin I polypeptides, one that is still membrane anchored but lacks the last 116 amino acids of the cytoplasmic domain and another that is released into the lumen of the ER and contains only the amino terminal 332 residues, are rapidly degraded soon after their synthesis without transfer to lysosomes. The degradation of these polypeptides began within 10 min of their synthesis and was unaffected by lysosomotropic agents and inhibitors of lysosomal proteases. Other authors have reported a similar nonlysosomal rapid degradation for unassembled polypeptide subunits of proteins that are normally transported to the cell surface. These include components of the T cell (Lippincott-Schwartz et al., 1988; Chen et al., 1988; Bonifacino et al., 1989; Wileman et al., 1990*a*), acetylcholine (Merlie et al., 1982) and asialoglycoprotein (Amara et al., 1989) receptors, and immunoglobulin chains (Sitia et al., 1990). In addition, various mutant polypeptides, such as a truncated variant of the α-subunit of β-hexosaminidase found in cultured fibroblasts from certain Tay-Sachs patients (Lau and Neufeld, 1989), a truncated influenza hemagglutinin molecule (Doyle et al., 1986), and an α globin chain that was introduced into the ER by an attached cleavable signal sequence (Stoller and Shields, 1989) were degraded by this process. In contrast to these cases, however, the completely translocated ribophorin I variant (RI_{332}) appeared to be degraded in two distinct pre-Golgi compartments, one which is the ER itself, or a portion of it, and a second to which the protein was transferred by vesicular flow. Thus, the degradation rate of RI_{332} was initially similar to that of the membrane-anchored variant (RI_{467}), but after ~50 min it increased more than threefold, and this increase was prevented by treatment with monensin or CCCP, ionophores which block protein transport from the ER to the Golgi apparatus (Tartakoff, 1983*a*,*b*; Argon et al., 1989). Moreover, CCCP did not affect the second phase of degradation when added after that phase had already begun.

Our finding that both monensin and CCCP prevented the occurrence of the second phase of degradation of RI_{332}, but did not reduce the first degradative rate, supports the notion that the initial phase of degradation takes place in the ER itself. This is consistent with other previous observations (Lippincott-Schwartz et al., 1988) that this ionophore, when applied at physiological pH, as done in our experiments, did not affect the monophasic rate of degradation observed for unassembled TCR subunits. The ionophores, therefore, are likely to act by preventing the flow of RI_{332} to a second compartment, rather than by preventing an enhanced degradation in the ER because they change the ionic conditions within this organelle. In this regard, it is worth noting that Lippincott-Schwartz et al. (1988) observed that the rapid nonlysosomal degradation of the TCR α chain began after a 20-min lag, which they interpreted as possibly reflecting the time required for the transfer of the newly synthesized protein to a degradative compartment. In that system, however, the ionophores apparently had little effect on the nonlysosomal degradation and it was concluded that vesicular movement was not involved in the transport. It is clear that in our case there was no extended lag before degradation began since 37% of the RI_{332} molecules were degraded with first order kinetics during the first 45–50 min.

The degradation of both truncated ribophorin I variants was prevented when the cells were incubated at 20°C, a temperature that allows exit of proteins from the ER to the Golgi apparatus (Saraste and Kuismanen, 1984) and, therefore, is also likely to permit flow of the truncated molecules from the first to the second degradative compartment if the latter is on the natural route from the ER to the Golgi apparatus. If this is the case, the stability of the truncated ribophorins at 20°C would indicate that the proteolytic processes that take place in the two pre-Golgi compartments are themselves extremely sensitive to temperature, which would be expected if the degradation is carried out by membrane-bound enzymes whose activity is affected by the fluidity of the membrane lipids (DePierre and Dallner, 1975; Mitropoulos et al., 1980). A similar inhibitory effect of low temperature on pre-Golgi degradation has been reported for other proteins (Chen et al. 1988; Stoller and Shields, 1989).

The finding that Ca^{2+} ionophores, in contrast to the proton/monovalent cation ionophores, almost completely blocked degradation of both truncated ribophorins is of considerable interest. The effect of the Ca^{2+} ionophores appears to be caused by the depletion of the ER calcium pool since it occurred even when EGTA was added to the medium. The Ca^{2+} concentration in the ER lumen has been estimated to be several orders of magnitude higher than in the cytoplasm (up to 3 mM, for review see Sambrook, 1990), and it has been postulated that the major luminal resident proteins of the ER, which have the capacity to bind large amounts of calcium, form a gel within the ER lumen that prevents their exit towards the Golgi apparatus (Booth and Koch, 1989; Sambrook, 1990). In some cell types, Ca^{2+} depletion induces the secretion of luminal ER resident proteins and this is thought to result either directly from the dissolution of the gel (Booth and Koch, 1989) or from an ensuing mass vesiculation of the ER membrane (Sambrook, 1990). It is, however, unlikely that the calcium ionophores prevented the degradation of the truncated ribophorins by inducing their exit from the degradative compartment(s) towards the Golgi apparatus, since our studies were carried out with HeLa cell transformants, and HeLa cells do not manifest the calcium ionophore-induced secretion of ER resident proteins (Booth and Koch, 1989). Moreover, in the presence of the Ca^{2+} ionophores, the truncated ribophorins were not secreted nor did they undergo posttranslational modifications by Golgi enzymes, which are observed when the cells are treated with BFA.[2] The most likely explanations for the effect of the Ca^{2+} ionophores are that, either the proteolytic enzymes that carry out the pre-Golgi degradation of RI_{332} require high levels of Ca^{2+} for their function, or Ca^{2+} depletion, by altering the conformation of the truncated ribophorin renders it resistant to the action of the proteases. The inhibitory effect of the Ca^{2+} ionophores that we observed is in marked contrast with the behavior of the unassembled α chain of the TCR in T cell hybridomas, which in the presence of a Ca^{2+} ionophore was found to be degraded by the nonlysosomal pathway (Lippincott-Schwartz et al., 1988), and with the behavior of TCR-β and CD3-δ chains in transfected cells, whose degradation was accelerated by the ionophore after a 1-h lag period (Wileman et al., 1991). We must conclude that, if the same enzymes are involved in the degradation of all these proteins, the differential effect of Ca^{2+} must reflect a differential effect of this ion on the conformation of the various proteins.

One of our most striking findings is that both phases of degradation of RI_{332} were completely inhibited by 1,10-phenanthroline, a metal chelator that inhibits Zn^{2+}-dependent metalloproteases (Chlebowski and Coleman, 1976). This strongly indicates that a metalloprotease, or a family of such enzymes, carries out the pre-Golgi degradative processes. Although the presence of a Zn^{2+}-dependent endopeptidase activity has been demonstrated in microsomes from several animal species (Mumford et al., 1980), the involvement of such an activity in protein degradation within the ER has not been reported previously.

The interpretation that the biphasic degradation of RI_{332}, indeed, reflects the participation of two distinct subcellular compartments is consistent with the effect of the macrolid antibiotic BFA. In cells treated with this drug, the degradation became monophasic and occurred with a rate intermediate between those of the two phases observed in the control cells. BFA is known to induce disassembly of the Golgi apparatus and a relocation of Golgi enzymes to the ER (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Doms et al., 1989; Ulmer and Palade, 1989). This relocation is thought to occur through an "intermediate" or a "salvage compartment" (see Pelham, 1989) that is normally traversed by proteins "en route" to the Golgi apparatus (Lippincott-Schwartz et al., 1990). If the second compartment of degradation of RI_{332} corresponds to the intermediate compartment, or is en route to it, it would be expected that BFA leads to an equilibration of the environment within that compartment with that of the ER itself.

Several inferences can be made from the behavior of the truncated ribophorins with respect to the features of the intact proteins that are responsible for their retention in that organelle or determine their turnover rates. The segregation within the ER of some transmembrane proteins, such as the adenovirus E19 glycoprotein (Pääbo et al., 1987), several forms of UDP-glucuronyl transferase (Nilsson et al., 1989; Jackson et al., 1990), and possible 3-hydroxy-3-methylglutaryl coenzyme A reductase (Jackson et al., 1990), appears to be due to the presence within the extreme carboxy-terminal segments of the polypeptides, which are exposed on the cytoplasmic side of the ER membrane. Such signals would determine either the retention of the proteins in the ER or the retrieval of "escaped" molecules from a post-ER "salvage" compartment. These carboxy-terminal signals show significant sequence variation but contain two essential lysine residues, one in the third position from the COOH terminus, and the other in the fourth or fifth position (Jackson et al., 1990). On the other hand, ribophorins I and II and a subunit of the signal peptidase (SPC22/23) (Shelness et al., 1988) do not contain retention signals of this type at their COOH termini. In fact, when added to reporter proteins that are normally transferred to the plasma membrane, the COOH-terminal segments of ribophorins do not lead to ER retention in the chimeric protein (Jackson et al., 1990). Although our experiments with the truncated ribophorin I molecules, one which lacks the extreme COOH-terminal segment and another which is not even membrane anchored, would be consistent with the notion that a signal that prevents the egress of ribophorin I from the ER is located in its luminal domain, it is quite possible that the truncated proteins are retained in the ER simply because they are misfolded and, therefore, are recognized by a system that effects quality control on newly synthesized polypeptides. The behavior of the truncated

ribophorins would then resemble that of truncated forms of viral envelope glycoproteins that are also retained in the ER (see Rose and Doms, 1988). It is noteworthy, however, that RI_{332} molecules were not associated with BiP, a protein that contributes to the retention of some misfolded proteins in the ER, and, therefore, their retention must involve some other mechanism.

Native ribophorin I is part of a protein network within the ER membrane that includes ribophorin II, in a 1:1 stoichiometric ratio with ribophorin I, and components of the translocation apparatus, such as the ribosome binding sites and the signal recognition particle receptor (Kreibich et al., 1978*a*,*b*; Marcantonio et al., 1984; Amar-Costesec et al., 1984; Yu et al., 1989). The normal retention of ribophorin I in the ER may, therefore, be a consequence of its incorporation into this network, which would prevent its entrance into the bulk flow carrier vesicles that exit from the ER (Wieland et al., 1987). If this is the case, the same sequences that mediate the incorporation of this protein into the network would, in effect, be serving as retention signals. Moreover, the stability of the native ribophorin would result from its incorporation into the network, which sequesters it from the degradative machinery, so that the truncated variants would be unstable simply because they lack the capacity to associate with the network. This would imply that, normally, assembled ribophorin molecules contain features that could be recognized by the degradative system but are not accessible to this system when the proteins are part of the network. Similar alternative explanations have been proposed (Bonifacino et al., 1990; Wileman et al., 1990*b*) to explain the instability of unassembled subunits of the TCR complex. For the α and β subunits of this complex (Bonifacino et al., 1990; Wileman et al., 1990*b*), as well as for unassembled transmembrane immunoglobulin heavy chains (Williams et al., 1990), however, it has been shown that the signals that lead to rapid degradation of these polypeptides are contained in their transmembrane domains and that, in the case of the α-subunit of the TCR, such signals are masked during multimerization, since the same domain directly mediates the association with other subunits (Manolios et al., 1990). In contrast to the examples just mentioned, our results with RI_{332} clearly show that a membrane anchor is not essential for rapid pre-Golgi degradation. A similar conclusion can be drawn from previous observations on the rapid turnover of other abnormal luminal protein molecules, such as α globin (Stoller and Shields, 1988) and mutant α_1-antitrypsin polypeptides (Le et al., 1990).

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