Ubiquitination Is Required for the Retro-translocation of a Short-lived Luminal Endoplasmic Reticulum Glycoprotein to the Cytosol for Degradation by the Proteasome*

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In the endoplasmic reticulum (ER), an efficient "quality control system" operates to ensure that mutated and incorrectly folded proteins are selectively degraded. We are studying ER-associated degradation using a truncated variant of the rough ER-specific type I transmembrane glycoprotein, ribophorin I. The truncated polypeptide (RI332) consists of only the 332 amino-terminal amino acids of the protein corresponding to most of its luminal domain and, in contrast to the long-lived endogenous ribophorin I, is rapidly degraded.

Here we show that the ubiquitin-proteasome pathway is involved in the destruction of the truncated ribophorin I. Thus, when RI332 that itself appears to be a substrate for ubiquitination was expressed in a mutant hamster cell line harboring a temperature-sensitive mutation in the ubiquitin-activating enzyme E1 affecting ubiquitin-dependent proteolysis, the protein is dramatically stabilized at the restrictive temperature. Moreover, inhibitors of proteasome function effectively block the degradation of RI332. Cell fractionation experiments indicate that RI332 accumulates in the cytosol when degradation is prevented by proteasome inhibitors but remains associated with the lumen of the ER under ubiquitination-deficient conditions, suggesting that the release of the protein into the cytosol is ubiquitination-dependent. Accordingly, when ubiquitination is impaired, a considerable amount of RI332 binds to the ER chaperone calnexin and to the Sec61 complex that could represent a candidate protein that might occur when the protein is still associated with the ER, as the trimmed glycoprotein intermediate efficiently interacts with calnexin and Sec61.

From our data we conclude that the steps that lead to the newly synthesized luminal ER glycoprotein to degradation by the proteasome are tightly coupled and that especially ubiquitination plays a crucial role in the retro-translocation of the substrate protein for proteolysis to the cytosol.

Most proteins of the endomembrane system as well as plasma membrane and secretory proteins are synthesized on polysomes bound to the membrane of the rough endoplasmic reticulum. During and shortly after their synthesis, the ectodomain of these polypeptides assume their three-dimensional conformation in the lumen of the ER,1 and the proteins may then also become part of oligomeric complexes. The ER houses an efficient "quality control system" to ensure that transport out of this organelle is limited to properly folded and assembled proteins (1, 2). Proteins that fail to assume their correct final conformation in the lumen of the ER, in most cases, do not stably remain in this compartment, rather they are degraded by a proteolytic system (3, 4).

An increasing number of diseases characterized by an "ER storage phenotype" results from impaired quality control of the ER (5). For instance, it has been observed that in most cases of cystic fibrosis, mutated forms of the transmembrane conductance regulator (CFTR) are not expressed at the cell membrane but are retained and degraded in the ER or a related compartment (6, 7). Similarly, α1-antitrypsin (α1-AT) deficiency patients with the Z mutation in α1-AT accumulate the mutant protein in the ER of hepatocytes (8, 9). In addition, some cases of familial hypercholesterinemia (10, 11) and Tay-Sachs disease (12) are also related to impaired transport out of the ER. The process, and possibly the mechanism(s) involved, of ER-associated degradation appears to be highly conserved in eukaryotes, as this phenomenon has also been observed in yeast (13–15).

In several cases it has been shown that substrate proteins for ER-associated degradation interact with chaperones present in the ER, which thus might be involved in the quality control process that leads to targeting of those proteins for degradation. For instance, the binding of CFTR (16) and of the PiZ variant of α1-AT (9, 17) to calnexin, a chaperone that recognizes glycoproteins in their mono-glucosylated forms in the ER (18, 19), has clearly been demonstrated. Furthermore, BiP that has been shown to bind to a variety of folding intermediates in the lumen of the ER could also represent a candidate protein that interacts with misfolded polypeptides that eventually are delivered for proteolysis. In fact, it appears that the time of

1 The abbreviations used are: ER, endoplasmic reticulum; ALLN, N-acetyl-i-leucyl-t-leucyl-t-norleucinal; α1-AT, α1-antitrypsin; BFA, brefeldin A; CFTR, cystic fibrosis transmembrane conductance regulator; CHAPS, 3-[3-cholamidopropyl]dimethylammonio-1-propanesulfonic acid; CHO, Chinese hamster ovary; CPY*, a mutant form of carboxypeptidase Y; endo H, endoglycosidase H; leupeptin, α1-antitrypsin-t-leucyl-t-leucyl-t-leucinal; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; PMSF, phenylmethylsulfonyl fluoride; RI332, a truncated form of ribophorin I containing its 332 NH2-terminal amino acids; MHC, major histocompatibility complex; TCR, T cell antigen receptor; TLCK, N-tosyl-L-lysine chloromethyl ketone; TPPC, N-tosyl-L-phenylalanine chloromethyl ketone; Tricine, N-tris(hydroxymethyl)methylglycine; ZLL, carboxbenzoxyl-t-leucyl-t-leucyl-t-leucinal; ZLLNva, carboxbenzoxyl-t-leucyl-t-leucyl-t-norvalinal.
interaction of a substrate protein with BiP correlates with its half-life (20, 21).

The major pathways of protein degradation in the eukaryotic cell include lysosomal proteolysis, ubiquitin-dependent lysosomal proteolysis, ubiquitin-independent proteasomal proteolysis, and ubiquitin-dependent proteasomal proteolysis (22–24). The latter plays a pivotal role in the rapid turnover of abnormal proteins and in the regulation of the steady state of a variety of proteins that include cyclins, kinases, tumor suppressors, and transcriptional regulators (23–25). In this case, ubiquitin, a small polypeptide of 76 amino acids, is activated by a ubiquitin-activating enzyme (E1) in a reaction that requires ATP hydrolysis. The activated ubiquitin molecule is then transferred to a ubiquitin-conjugating enzyme (E2) that catalyzes the formation of an isopeptide bond between the COOH-terminal glycine of ubiquitin and the ε-amino group of a lysine residue on target proteins. The mono-ubiquitinated substrates then undergo further ubiquitinations via the lysine residue at position 48 of ubiquitin, leading to the formation of multi-ubiquitin chains that target proteins to degradation by the 26 S proteasome (23, 24).

In recent years it has become clear that soluble and integral membrane proteins that have been targeted to the ER are, in fact, degraded by the ubiquitin-proteasome pathway. By using a genetic approach, Hiller et al. (26) provided evidence that the degradation of a variant of carboxypeptidase Y (CPY*) that is retained in the ER of yeast depends on the activity of Ubc6p, an ER-bound ubiquitin-conjugating enzyme, as well as functional proteasomes. Furthermore, it was demonstrated that retrograde transport of CPY* from the ER to the cytosol depends on ubiquitination, in which a complex is involved that consists of the two ubiquitin-conjugating enzymes, Ubc6p and Ubc7p, and Cue1p, an ER transmembrane protein required for the recruitment of Ubc7p to the ER membrane (27). Moreover, Wiertz et al. (28) observed an interaction between MHC class I heavy chain molecules and Sec61, suggesting that retrograde transport of substrate proteins to the cytosol may occur through the translocation channel. This view was strengthened by the recent finding that certain mutant yeast Sec61 alleles are defective in the export out of the ER of substrate proteins for degradation (29, 30).

We are studying ER-associated degradation using a COOH-terminally truncated variant of ribophorin I, a type I ER transmembrane glycoprotein that is a component of the oligosaccharyltransferase complex (31–33). When the mutant protein, RI332, that contains only the NH2-terminal 332 amino acids of the luminal domain of ribophorin I is expressed in permanent transformants of HeLa cells, it is rapidly degraded by a non-lysosomal pathway with biphasic kinetics. The first phase of degradation is characterized by a half-life of about 1 h and is followed, after approximately 45 min, by a second phase of 3-fold accelerated degradation. In contrast, endogenous ribophorin I is very stable and has a half-life of more than 24 h (34, 35). Here we show that the ubiquitin-proteasome pathway is involved in the degradation of RI332 and that, in fact, release to the cytosol of the substrate protein for degradation and ubiquitination are tightly coupled.

MATERIALS AND METHODS

Reagents—The mammalian expression vector pCI-neo was purchased from Promega (Madison, WI); maleimide-activated keyhole limpet hemocyanin was from Pierce, and protein A-Sepharose CL-4B beads were from Pharmacia (Uppsala, Sweden). Geneticin (G418 sulfate), α-minimal essential medium, methionine-free RPMI 1640, other cell culture components, and Lipofectin were from Life Technologies, Inc. Trypsin is from bovine pancreas, BFA, aprotinin, leupeptin, l-1-phenyl-

Nva were from Peptides International (Louisville, KY). Endo H and ALLN were obtained from Boehringer Mannheim Biochemicals (Mannheim, Germany). Met-[^35]S-Label containing [35S]methionine was purchased from American Radiolabeled Chemicals (St. Louis, MO). The proteasome inhibitors ALLN, ZLLL, and ZLL-Nva were dissolved in DMSO and kept as stock solutions at −20 °C.

Antibodies—The polyclonal rabbit antibody against rat liver ribophorin I was a generous gift from Dr. Gert Kreibich (New York University School of Medicine) and has been described previously (34, 36, 37). The polyclonal rabbit anti-Sec61α antibodies (38) and anti-PI antibodies were kindly provided by Dr. Tom A. Rapoport (Harvard Medical School, Boston) and Dr. Donald C. Pearson (Bristol-Myers Squibb, Princeton, NJ), respectively. The monoclonal mouse CTR433 antibody, a marker for the medial Golgi cisternae (39), was a gift from Dr. Michel Bornens (Institut Curie, Paris, France). The polyclonal anti-calnexin antibody is directed against the COOH-terminal peptide of calnexin (amino acids 555–573 of the mature dog protein) (Ref. 40). The peptide that contains an additional cysteine residue at the NH2-terminal side was coupled to maleimide-activated keyhole limpet hemocyanin and injected into a rabbit for antibody production (see also Ref. 41). A polyclonal rabbit anti-ubiquitin antibody was purchased from StressGen Biotechnologies (Victoria, Canada). Affinity purified, Texas Red-conjugated goat anti-mouse F(ab')2-IgG was obtained from Accurate Chemicals (Westbury, NY).

Cell Culture and Transfections—E36 and ts20 cells were kindly provided by Dr. Alan L. Schwartz (Washington University School of Medicine) (42). The cells were grown at 30 °C in α-minimal essential medium, supplemented with glucose (4.5 g/liter), 10% fetal calf serum, penicillin G (100 IU/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (250 ng/ml). The generation of the cDNA coding for the 332 NH2-terminal amino acids of rat ribophorin I (RI332) and its cloning into the mammalian expression vector pCI-neo will be described elsewhere (see also Refs. 32 and 34). The cells were transfected with the expression construct by the Lipofectin method according to the manufacturer's instructions, using 1 μg of DNA and 10 μl of Lipofectin reagent on cells cultured in a 6-cm dish and an incubation time of 18 h. Permanent transformants of E36 and ts20 cells expressing RI332 (designated E36-RI332 and ts20-RI332 cells) were obtained after selection for growth in the presence of geneticin (600 μg/ml). Single clones of highly expressing cells were selected, cultured in the continued presence of geneticin (300 μg/ml), and used further for the experiments performed during this study.

Treatment of Cells with Proteasome Inhibitors, Temperature Conditions, Cell Labeling, and Immunoprecipitations—The transfected E36-RI332 and ts20-RI332 cell cultures were grown in 35-mm dishes to confluence (5–8 × 104 cells per dish) for pulse-chase experiments at the restrictive temperature, the cells were preincubated at 41 °C for 2 h. For treatments with proteasome inhibitors, the cells were pretreated at 30 or at 41 °C with ALLN (80 μM), ZLL-Nva (40 μM), or ZLL (50 μM) for 90 min in complete medium and for another 30-min period in serum- and methionine-free RPMI 1640 medium. Cells were labeled in serum- and methionine-free medium containing [35S]methionine (250 μCi/ml) for 10 min at the indicated temperatures. Subsequent chase incubations were carried out in complete medium supplemented with methionine (5 μM). Cells were lysed with 300 μl of an SDS-containing buffer (25 mM Tris-HCl, pH 7.4, 95 mM NaCl, 3 mM EDTA, 2% SDS, and a mixture of the following protease inhibitors: 1.7 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 μM PMSEF), and after sonication and boiling of the cell lysate, 1 ml of wash buffer (25 mM Tris-HCl, pH 7.4, 95 mM NaCl, 3 mM EDTA, 0.2% SDS, 1.25% Triton X-100, protease inhibitors as above) was added. Immunoprecipitations were performed with anti-ribophorin I antiserum (4 μl/ml lysisate) and analyzed by SDS-PAGE using 8% gels, unless noted otherwise, and fluorography, as reported previously (34). When necessary, immunoprecipitates were treated with endo H, as described (43). Quantitations of immunoprecipitations were performed by scanning densitometry using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Indirect Immunofluorescence—ts20-RI332 cells were grown on coverslips for 36 h at 30 °C. After pretreatment of the cells at the appropriate temperature conditions, followed by an incubation in the absence or presence of BFA (5 μg/ml) for 30 min, the cells were fixed with 100% methanol at −20 °C for 30 min and then prepared for immunofluorescence staining, as described previously (44). The monoclonal CTR433 mouse antibody was used as a marker for the medial Golgi cisternae (39) and applied at a dilution of 1:2 in blocking medium (1% non-fat dry milk in phosphate-buffered saline). The secondary affinity purified, Texas Red-conjugated goat anti-mouse F(ab')2 antibody fragments were used at a dilution of 1:40 in the same medium. After mounting, the
staining was visualized on a Zeiss Axiosvert 135 photomicroscope (Carl Zeiss, Oberkochen, Germany) equipped with epifluorescence optics and photographed using Kodak TMAX-400 ASA film.

**Sequential Immunoprecipitations with Anti-ribophorin I and Anti-ubiquitin Antibodies—**E36-RI332 cells were grown in a 6-cm dish near to confluence (approximately 5 × 10^6 cells). The supernatants were loaded on a 2 M sucrose cushion containing 25 mM Tris-HCl, pH 7.6, 5 mM EDTA, and protease inhibitors and then overlaid with isotonic buffer described above. The samples were centrifuged in a Beckman SW60 rotor at 110,000 × g for 16 h. The supernatant fractions were subjected to protein A-Sepharose chromatography as described previously (34). The immunoprecipitates were analyzed by SDS-PAGE and fluorography as described (34).

**RESULTS**

To characterize the pathway involved in the degradation of R_{332}, we established permanent transformants of CHO-E36 and CHO-ts20 cells that express the protein. E36 is the wild type cell line, and ts20 is the corresponding mutant that expresses a thermolabile ubiquitin-activating enzyme E1 (42). At temperatures above 40 °C, the ubiquitin system and consequently its protein-ubiquitin conjugating capacity are inactivated to less than 10%. We compared the life cycle of R_{332} at the permissive temperature (30 °C) and at the non-permissive temperature (41 °C) in both cell lines (Fig. 1).

R_{332} was rapidly degraded at the permissive temperature in E36-RI332 cells so that after 60 min of chase no band was detectable on the gel, whereas in ts20-RI332 cells the protein was degraded somewhat more slowly, but also in this cell line only a very small amount of R_{332} was recovered after 60 min of chase (Fig. 1A).

As to the degradation at 41 °C in both cell lines, it is noteworthy that heat treatment of many cells including E36 leads to stress-induced degradation of many cellular proteolysis substrates, but heat treatment of ts20 cells inactivates the ubiquitin-conjugating system (42). At the non-permissive temperature, the inactivation of ubiquitination strongly prevented the degradation of R_{332} in ts20-RI332 cells, as a substantial fraction of the original amount of the protein persisted even after 3 h and 30 min of chase (Fig. 1C). As expected, R_{332} was rapidly degraded in E36-RI332 cells at 41 °C (Fig. 1B).

It is interesting to mention that R_{332} molecules synthesized at 41 °C in ts20-RI332 cells remain in a state susceptible to degradation, which occurs as soon as the incubation temperature is changed to 30 °C (Fig. 1D). This observation may be due to the fact that the cells rapidly regain their ubiquitin-conjugating capacity under these conditions, thus allowing for efficient ubiquitin-dependent degradation of the protein. When the proteasome activity was blocked by the addition of TPCK, TLCK (500 μg/ml each), and PMSF (5 mM), degradation of the protein at 41 °C was significantly slowed. These results demonstrate that ubiquitination is required for the degradation of R_{332} in ts20-RI332 cells at 41 °C.
Fig. 1. The truncated ribophorin I variant, RI₃₃₂, is stabilized in ts20-RI₃₃₂ cells at the non-permissive temperature. The E36 and ts20 cell transformants expressing RI₃₃₂, plated in 35-mm dishes to a density of 5–8 × 10⁶ cells per dish, were kept at 30 °C (A) or preincubated at 41 °C for 2 h (B–D). Then the cells were incubated in serum- and methionine-free medium for 30 min and pulse-labeled with the same medium containing [³⁵S]methionine (250 μCi/ml) for 10 min at the temperatures indicated. Subsequent chase incubations were carried out in complete medium supplemented with 5 mM unlabeled methionine for up to 90 min (A) or 210 min (B–D) at 30 °C (A and D) or 41 °C (B and C). Cells were lysed with an SDS-containing buffer and processed for immunoprecipitation with a polyclonal rabbit anti-ribophorin I antibody. The samples were analyzed by SDS-PAGE (8% gels) and fluorography. RI indicates the position of the native ribophorin I.

ER transport (48); hence, the ability of ts20-RI₃₃₂ cells to support BFA-induced relocation of a Golgi protein to the ER at 41 °C was investigated. Indirect immunofluorescence labeling using the CTR433 antibody as a marker for the medial cisternae of the Golgi apparatus (39) on ts20-RI₃₃₂ cells at both 30 and 41 °C resulted in a typical Golgi staining (Fig. 2, A and C). Conversely, after treatment of the cells with BFA for 30 min, a fluorescence pattern characteristic for the ER was obtained at both temperatures (Fig. 2, B and D). This result indicates that BFA-induced retrograde Golgi to ER transport remains functional in ts20-RI₃₃₂ cells at the non-permissive temperature. Furthermore, it is apparent that RI₃₃₂ must have been essentially fully translocated into the lumen of the ER, as it receives its N-linked oligosaccharide at Asn²⁷⁷ (see below, Fig. 4). These observations, together with the result described in Fig. 1D, indicate that several cellular processes related to the endomembrane system are not affected to any significant extent by incubation of the ts20-RI₃₃₂ cell mutant at 41 °C under the conditions used in this study.

To investigate if the proteasome is involved in the rapid turnover of RI₃₃₂, we observed the effect of different proteasome inhibitors such as ALLN (MG101), ZLLNva (MG115), and ZLLL (MG132) on the degradation of RI₃₃₂ in E36-RI₃₃₂ cells at 30 °C (Fig. 3A) and at 41 °C (B) as well as of ts20-RI₃₃₂ cells at 30 °C (C). All three of these proteasome inhibitors markedly blocked RI₃₃₂ degradation, since a consistent amount of the protein was recovered after 2 h of chase.

Interestingly, two additional forms migrating approximately 1.5 to 2 kDa below the band corresponding to RI₃₃₂ appeared in the immunoprecipitates of E36-RI₃₃₂ cells incubated with proteasome inhibitors after 45 min of chase. Since it has been shown that several glycoproteins undergo deglycosylation prior to degradation, such as the heavy chain of the MHC class I molecules (28), we speculated that the newly arising band might correspond to deglycosylated forms of RI₃₃₂. Ribophorin I as well as RI₃₃₂ contain three potential N-glycosylation sites, but only one of these is used by oligosaccharyltransferase. It has been shown in earlier work that RI₃₃₂ remains endo H-sensitive throughout its lifetime (43). Taking this premise into account, we performed an endo H digestion on anti-ribophorin I immunoprecipitates from E36-RI₃₃₂ and ts20-RI₃₃₂ cells incubated under various conditions (Fig. 4). As expected, after endo H digestion only a single band was detectable (RI₃₃₂*; lanes a, d, g, and j), migrating at the same position as the lowest form of RI₃₃₂ recovered from the undigested immunoprecipitates (lanes a, d, g, and j). This finding suggests that the additional forms of RI₃₃₂ appearing after proteasome inhibitor treatment represent the fully deglycosylated protein (RI₃₃₂*) as well as a species where the single N-glycan moiety has been trimmed. The latter form should, therefore, correspond to a trimmed glycoprotein intermediate (RI₃₃₂*). Although we did not establish the sugar composition of the oligosaccharide structure of this intermediate, these results indicate that endo H is capable of acting on truncated N-glycans. In fact, it has been shown previously that endo H cleaves trimmed N-glycans that retain an α1,6-linked core oligosaccharide, whereas the truncated glycans detected on glycoproteins in a CHO cell mutant bearing a glycosylation and temperature-sensitive secretion defect, for example, were found to be endo H-resistant (49). It is noticeable that the trimmed glycoprotein intermediate of RI₃₃₂ accumulates over time when proteasomal degradation is inhibited by ZLLL (Fig. 3, lanes j–l). On the other hand, the fact that
the lower band of the doublet migrating faster than RI332 is observed at 10 min of chase (lane j) could be explained by newly synthesized RI332 molecules that have never been N-glycosylated by oligosaccharyltransferase during the labeling period. To determine directly whether RI332 itself is a substrate for ubiquitination, co-immunoprecipitation experiments of ribophorin I and ubiquitin from a lysate of E36-RI332 cells were performed. The proteasome inhibitor ZLLL was included during the pulse-chase incubations, and NEM, an isopeptidase inhibitor, was included in the immunoprecipitation buffers to accumulate proteasome substrates and to maintain them in their poly-ubiquitinated state. The cell lysate was used for a co-immunoprecipitation experiment as follows. Material immunoprecipitated by antibodies directed against ribophorin I was reprecipitated with anti-ubiquitin antibodies (Fig. 5, lane d) or vice versa (lane c). When RI332 was immunoprecipitated from cell lysates of ZLLL-treated cells in the presence of 0.2% SDS and 1% Triton X-100, and in the absence of NEM, only bands between 38 and 36 kDa were detectable (lane a; see also Fig. 3). However, in subsequent immunoprecipitations with anti-ribophorin I and anti-ubiquitin antibodies (Fig. 5, lanes c and d), or even in the anti-ribophorin I immunoprecipitations alone (lane b), all under the same conditions but in the presence of NEM, higher molecular weight bands became evident representing ubiquitinated forms of RI332. In fact, it appears that the majority of the RI332 molecules is ubiquitinated under the experimental conditions used, as in the presence of NEM the band corresponding to unubiquitinated RI332 almost disappears (lane b). As expected, from the immunoprecipitation with anti-ubiquitin antibody alone, a number of higher molecular weight bands became discernible (lane e).

Considering that the ubiquitin-proteasome pathway is located in the cytoplasm, we wanted to investigate the intracellular distribution of RI332 molecules when their degradation is inhibited. To this effect, a cell fractionation experiment was performed in which ribophorin I and RI332 were immunoprecipitated from the membrane and the cytosolic fractions of E36-RI332 cells (Fig. 6a) and ts20-RI332 cells (Fig. 6d) labeled for 30 min and incubated in chase medium under different conditions. Only a small portion (12%) of RI332 was detected in the cytosol of untreated E36-RI332 cells at 10 min of chase (Fig. 6A, lanes a and b). In the presence of ZLLL, the total amount of
ri332 recovered from E36-RI332 cells at 10 min of chase increased slightly (to 17%) when compared with untreated cells, as the protein becomes stabilized after inhibition of the proteasome (compare lanes e and f with a and b). In ts20-RI332 cells, a portion of RI332 (16 and 19%, respectively) was recovered from the cytosol after 10 min of chase at 30 and 41 °C (Fig. 6B, lanes a and b as well as e and f).

Strikingly, after extension of the chase time to 1 h, a significantly increased amount (61%) of RI332 was recovered from the cytosol of ZLLL-treated E36-RI332 cells (Fig. 6A, lanes g and h), indicating that the retro-translocated protein accumulates in the cytosol when the function of the proteasome is compromised. In contrast, RI332 was not detectable in the cytosol of ts20-RI332 cells at 41 °C after 1 h of chase (Fig. 6B, lane h) indicating that the small portion recovered at the 10-min chase time point must have been degraded, most likely due to the accessibility of RI332 to exogenously added protease.

To ascertain that RI332 indeed remains associated with the ER of ts20-RI332 cells at 41 °C and does not form aggregates in the cytoplasm, a cell fractionation experiment was performed in which membrane vesicles and high molecular weight protein complexes and aggregates were separated. To this aim, total membrane fractions prepared from radiolabeled ts20-RI332 cells grown at 30 °C or preincubated at 41 °C were subjected to ultracentrifugation over a 2 M sucrose cushion for 16 h. Under the conditions used, membranes float on top of this cushion, whereas protein complexes and also aggregates are sedimented (51). Ribophorin I and RI332 were immunoprecipitated from the interface above the 2 M sucrose cushion and from the pellet (Fig. 7). It is clearly evident that the majority (more than 95%) of RI332 is present in the membrane fraction at 41 °C and after 10 min (lane c) and 60 min (lane e) of chase, indicating that under ubiquitination-deficient conditions the protein remains membrane-associated for extended periods. At 30 °C, RI332 is also recovered from the membrane fraction shortly after its synthesis (lane a). These findings strongly suggest that RI332 does not form cytosolic aggregates when ubiquitination is impaired.

Furthermore, it was of interest to determine if RI332, when associated with microsomes, is contained within their lumen or facing the cytosolic side of the membrane. For this purpose, the accessibility of RI332 to exogenously added protease was assessed. Total membrane fractions prepared from radiolabeled ts20-RI332 cells grown at 30 °C (Fig. 8A) or preincubated at 41 °C (Fig. 8B) were treated with increasing concentrations of trypsin in the absence or presence of Triton X-100 for 30 min at 30 °C. When the detergent was omitted, in all cases RI332 remained protected to a large extent from exogenously added protease.
To analyze the role of calnexin in the quality control of the glycoprotein RI₃₃₂, we performed pulse-chase and co-immunoprecipitation experiments using anti-ribophorin I and anti-calnexin antibodies under different conditions that block RI₃₃₂ degradation (Fig. 9). In Fig. 9A, an experiment is shown to visualize the patterns obtained with ts20-RI₃₃₂ cells at 41 °C and at the 10-min chase time point: lanes a and f, anti-ribophorin I and anti-calnexin immunoprecipitations, respectively, under stringent conditions; lanes b and e, anti-ribophorin I and anti-calnexin immunoprecipitations, respectively, under non-stringent conditions; lanes c and d, co-immunoprecipitations in which first calnexin and ribophorin I, respectively, were immunoprecipitated, followed by anti-ribophorin I and anti-calnexin immunoprecipitations, respectively, under stringent conditions. Analogous experiments were performed for each cell line, chase time point, and condition. B and C, only ribophorin I and RI₃₃₂ immunoprecipitated under stringent conditions from anti-ribophorin I immunoprecipitations performed under non-stringent conditions (comparable to lane c in A) are shown for each cell line, chase time point, and condition. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. (Exposure times: A: lanes a–c, e, and f; 2 days; lane d, 20 days; B: 10 days; C: 20 days.)
Fig. 10. RI332 interacts with Sec61. ts20-RI332 cells were pulse-labeled for 30 min and chased for 10 min (lanes a and c) or 1 h (lanes b and d) at 30 °C in the absence or presence of ZLLL (50 μM) and at 41 °C in the absence of ZLLL (lanes e and f). For each condition and chase point a co-immunoprecipitation was performed in which Sec61β was immunoprecipitated first under non-stringent conditions in the presence of CHAPS (1%), and in a second round ribophorin I and RI332 were immunoprecipitated under stringent conditions in the presence of SDS (0.6%) and Triton X-100 (1%). For comparison, ribophorin I and RI332 were immunoprecipitated under stringent conditions from a cell lysate of ts20-RI332 cells incubated at 41 °C (lane g). Immunoprecipitates were analyzed on Tricine gels followed by fluorography. Only the ribophorin I and RI332 recovered from Sec61 β immunoprecipitations are shown. (Exposure times of lanes a–d, 10 days; lanes e–g, 1 day.)
Since ribophorin I and RI322 are N-glycosylated proteins, an interaction of these proteins with the ER chaperone calnexin is expected to occur during an early step in the quality control process to which these proteins are subjected. In fact, such an interaction has been demonstrated for several substrate proteins of ER-associated degradation, such as MHC class I heavy chains (61), CFTR (16), and the PiZ variant of \( \alpha_1 \)-AT (9). In our experiments, an association of calnexin with RI322 was clearly detectable only when the latter one was stabilized. Strikingly, however, large amounts of RI322 that remained essentially constant over time were found in interaction with the chaperone solely under ubiquitination-deficient conditions. These results are plausible considering that under these conditions retro-translocation is compromised, so that the polypeptide may accumulate in the lumen of the ER where its N-glycan stays accessible for prolonged binding to and release from calnexin. During this time of retention in the ER, UDP-glucose:glycoprotein glucosyltransferase may be involved in the monitoring of the progress the glycoprotein has made in its folding process, reglucosylate its N-linked oligosaccharide as soon as it has lost its remaining glucose residue due to the action of glucosidase II, and thus allow for several rounds of re-association of the glycoprotein with calnexin (62, 63).

As to the glycosylation status of RI322 recovered in complexes with calnexin, it is interesting to note that not only the completely glycosylated protein but also the partially deglycosylated form interacts with the chaperone. This finding is in support of the view discussed above that deglycosylation may be initiated within the ER, whereas the alternative possibility that the trimming intermediate is exposed at the cytoplasmic side of the ER membrane while still interacting with calnexin would be difficult to conceptualize. It should be stressed that the first step of deglycosylation appears to be ubiquitination-independent, as the partially deglycosylated form of RI322 is efficiently recovered from ts20-RI322 cells under restrictive temperature conditions. The interaction of this intermediate form of RI322 with calnexin could be explained if the partially trimmed oligosaccharide is capable of binding to the chaperone in the lumen of the ER. Partially trimmed high mannose oligosaccharides have indeed been found to be recognized by calnexin and its soluble homolog in the ER lumen, calreticulin (64, 65). Recently, it has been proposed that the post-translational trimming of N-linked oligosaccharides on glycoproteins that could be effected by ER mannosidases precedes the release of these proteins from calnexin and their subsequent intracellular degradation (66). A decrease in the degradation rate of CPY* has also been observed in a yeast strain in which the gene encoding the ER-associated \( \alpha_1 \)2-mannosidase has been disrupted (67). Alternatively, it could also be possible that calnexin binds to protein determinants on RI322, at least during a later phase of their interaction. Direct recognition of non-glycosylated domains of proteins by calnexin, although previously suggested, has recently been less favored, as it has been shown that the chaperone is able to bind to both folded and unfolded forms of N-glycosylated ribonuclease, which has been taken as strong evidence that calnexin acts exclusively as a lectin (68, 69).

Although ubiquitination of RI322 has been observed during this study, it remains to be determined whether additional factors involved in ER-associated degradation require ubiquitination. In this context, the ubiquitination of calnexin has been implicated in the degradative pathway of the PiZ variant of \( \alpha_1 \)-AT (9). From our observation that inhibition of ubiquitination results in prolonged interaction of RI322 and calnexin as well as impaired retro-translocation to the cytosol, ubiquitination of calnexin could provide a mechanism to trigger the release of the protein from the ER membrane.

During the passage of RI322 to its site of degradation, it is plausible that the protein is in close contact with the Sec61 complex, one of the major components of the translocation apparatus (70–72). This step in the degradation pathway of RI322 may be predicted, as it has been demonstrated that MHC class I heavy chain molecules are co-immunoprecipitated with antibodies directed against the \( \beta \) subunit of Sec61 (28). Further support for a role of Sec61 in the retro-translocation to the cytosol has been recently obtained, when it was shown that misfolded secretory proteins accumulate in the ER of yeast cells that express certain conditional sec61 alleles (29, 30). In agreement with these findings, we detected an association of RI322 with Sec61 in ts20-RI322 cells. A large portion of RI322 was recovered from Sec61 immunoprecipitates only when ubiquitination is blocked, whereas the interaction of RI322 with Sec61 was weakly detectable in control and proteasome inhibitor-treated cells. It appears that both completely glycosylated and partially deglycosylated forms of RI322 are detected in association with the translocation channel. In accordance with the cell fractionation experiments, these observations indicate that the integrity of the ubiquitination pathway may play a crucial role in the export of proteins from the ER lumen to the cytoplasm. At present, it is not clear, however, whether truly cytoplasmic proteasomes affect the degradation of ER protein substrates \textit{in vivo} or if proteasome particles associated with the ER membrane that have been detected by immunocytochemical means (73) perform this task. From our observation that RI322 accumulates in the cytosol of proteasome inhibitor-treated cells, the former possibility may seem more likely.

Taken together, our data are in support of the following model for ER-associated degradation of aberrant luminal glycoproteins. After translocation into the ER, signal peptide cleavage, and N-glycosylation, the carbohydrate moiety of the protein is recognized in its monoglycosylated form by the ER chaperone calnexin and possibly its soluble counterpart, calreticulin, which participate in the quality control process the protein is subjected to in the ER lumen. Upon completion of the quality control attempts that may involve several cycles of binding to and release from calnexin/calreticulin due to the cleavage of the remaining glucose residue by glucosidase II and reglucosylation of the N-linked oligosaccharide by UDP-glucose:glycoprotein glucosyltransferase, the protein is retro-translocated to the cytosol via the Sec61 channel of the translocation apparatus. The efficiency of the latter step, \textit{i.e.} the release of the polypeptide into the cytoplasmic space is strongly dependent on a functional ubiquitination pathway, as in the absence of ubiquitination the protein remains restricted to the lumen of the ER. Once in the cytoplasm, the protein is eventually delivered for degradation by the proteasome. During its metabolic fate, the protein is deglycosylated in two discernible steps, the first of which may occur within the ER lumen leaving the interaction of the protein with calnexin intact, whereas the second step may be accomplished by a cytosolic N-glycanase.

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\textbf{Note Added in Proof}—While this manuscript was under revision, a paper appeared (74) that provides further support for the conclusions.
drawn from this work. It was reported there that CPY*, a substrate for ER-associated degradation, accumulates in the ER lumen of yeast when UbCp- and UbCp-mediated ubiquitin conjugation is abolished, thus demonstrating directly the requirement of ubiquitination at the ER membrane for retro-translocation of CPY* to the yeast cytoplasm.

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