Human Endoplasmic Reticulum Mannosidase I Is Subject to Regulated Proteolysis*

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In the early secretory pathway, opportunistic cleavage of asparagine-linked oligosaccharides by endoplasmic reticulum (ER) mannosidase I targets misfolded glycoproteins for dislocation into the cytosol and destruction by 26 S proteasomes. The low basal concentration of the glycosidase is believed to coordinate the glycocalyx with prolonged conformation-based ER retention, ensuring that terminally misfolded glycoproteins are preferentially targeted for destruction. Herein the intracellular fate of human ER mannosidase I was monitored to determine whether a post-translational process might contribute to the regulation of its intracellular concentration. The transiently expressed recombinant human glycosidase was subject to rapid intracellular turnover in mouse hepatoma cells, as was the endogenous mouse ortholog. Incubation with either chloroquine or leupeptin, but not lactacystin, led to intracellular stabilization, implicating the involvement of lysosomal acid hydrolases. Inhibition of protein synthesis with cycloheximide led to intracellular depletion of the glycosidase and concomitant ablation of asparagine-linked glycoprotein degradation, confirming the physiologic relevance of the destabilization process. Metabolic incorporation of radiolabeled phosphate, detection by anti-phosphoserine antiserum, and the stabilizing effect of general serine kinase inhibition implied that ER mannosidase I is subjected to regulated proteolysis. Stabilization in response to genetically engineered removal of the amino-terminal domain of ER mannosidase I was monitored to determine whether a post-translational checkpoint contributes to the establishment of an equitable glycoprotein quality control standard by which the efficiency of asparagine-linked glycoprotein conformational maturation is measured.

Genetic information is directly transformed into biological activity in response to the correct conformational maturation and deployment of encoded proteins (1, 2). Arguably, this dual intention is best exemplified in the endoplasmic reticulum (ER)2 into which nascent secretory and membrane proteins are translocated. The adoption of native protein structure, facilitated by transient physical engagement with one or more molecular chaperones, precedes productive export from the ER (3). Rather than clogging the secretory pathway, molecules incapable of structural maturation are eliminated by a process coined “ER-associated degradation” (ERAD). The associated multiple requisite steps culminate in the dislocation of misfolded proteins into the cytosol for degradation by 26 S proteasomes (4, 5). Polyubiquitination functions as the signal that mediates both dislocation and proteolysis (Fig. 1) (6, 7).

As with glycoprotein folding (8), the earliest events of ERAD are orchestrated by the covalent modification of asparagine-linked oligosaccharides (Fig. 1). The capacity of general α-1,2-mannosidase inhibition to abrogate the elimination of numerous misfolded asparagine-linked glycoproteins (2, 9–19) and the effect of gene deletions in yeast (20) implicated ER mannosidase I as a key component in glycoprotein ERAD (GERAD).

Intracellular degradation of terminally misfolded α1-antitrypsin is accelerated in response to an experimentally elevated concentration of the recombinant glycosidase (18, 21). Its contribution toward a stochastically based substrate discrimination process was strengthened by destabilization of newly synthesized wild type α1-antitrypsin and transferrin under these conditions (18). A luminal bipartite signal consisting of the cleaved glycans and nonnative protein structure is suspected to function as the initial degradation signal. The very low basal concentration of ER mannosidase I (18) is proposed to preferentially target terminally misfolded glycoproteins for ERAD by coordinating the glycan modification with prolonged conformation-based ER retention. The subsequent trajectory of tagged molecules toward the ER dislocon is suspected to result from downstream machinery capable of recognizing the bipartite signal anatomy (Fig. 1).

The complementary nature by which the opposing systems of glycoprotein folding and quality control operate might function as a post-translational checkpoint in eukaryotic genome expression. Despite the fundamental role in which the overall process contributes to normal cell biology and possible involve-

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2 The abbreviations used are: ER, endoplasmic reticulum; ERManI, endoplasmic reticulum mannosidase I; ERAD, ER-associated degradation; GERAD, glycoprotein ERAD; GFP, green fluorescent protein; h, human; WT, wild type; NT, tail-less.
Human ERMan I Checkpoint Control

![Diagram](Image)

FIGURE 1. Conformational maturation and intracellular disposal of newly synthesized asparagine-linked glycoproteins represent a conformation-based branch point in the early secretory pathway. Modification of asparagine-linked oligosaccharides by glucosidase II (GlcII) and UDP-Glc:glycoprotein glucosyltransferase (UGGT) facilitates rounds of physical engagement with glycoprotein folding machinery. Conformational maturation (solid sphere) precedes productive transport and deployment. In contrast, nonnative protein structure, in combination with glycans cleaved by ERManI, generates a proposed luminal bipartite signal for recruitment to the ER dislocon. The low basal ERMan I concentration underlies a quantitative trait by which asparagine-linked glycoproteins are selected for destruction on the basis of inefficient conformational. The latter parameter is designated by opportunistic cleavage of asparagine-linked oligosaccharides in response to prolonged conformation-based retention in the ER. Dislocation into the cytosol and elimination by 26 S proteasomes are mediated by polyubiquitination.

- mannose
  + [Uba]_n
  ERMaI
  - glycan-mediated
  Rate-limiting & stochastic
  Signal formation
  Signal recognition
  Recruitment to dislocon
  Dislocation into cytosol
  Proteosomal degradation
  Glc II
  UGGT

EXPERIMENTAL PROCEDURES

Reagents—Unless stated otherwise, routine chemicals and buffers were purchased from Sigma. Lactacystin was purchased from the E. J. Corey laboratory (Harvard Medical School). Kifunensine was from Toronto Research Chemicals, Inc. Lipopectamine 2000 reagent was from Invitrogen. Brefeldin A was purchased from EPICENTRE (Madison, WI). The murine hepatoma cell line, Hepa 1a, was a kind gift from Dr. Gretchen Darlington (Baylor College of Medicine, Houston, TX). The establishment of Hepa1a (H1A) cells stably transfected with the null(Hong Kong) variant of human α1-antitrypsin (cell line H1A/N13) was previously reported (25).

Antisera—An immunoglobulin fraction of rabbit polyclonal anti-human α1-antitrypsin was purchased from Roche Applied Science. A monoclonal anti-phosphoserine antiserum was purchased from Sigma. Rabbit antisera ERMI-cc20 (18) was used for the detection of endogenous mouse ERMan I in untransfected Hepa1a cells. The transfected human homolog (hERMan I; accession number NM_007230) was detected following transfection with antisera (polyclonal anti-ER Man I antibody) under conditions that failed to detect the endogenous murine ortholog. The polyclonal antisera was generated against the purified recombinant catalytic domain (residues 241–699) expressed in Pichia pastoris (26). Approximately 200 μg (in 0.5 ml of phosphate-buffered saline) of the purified enzyme emulsified with an equal volume of Freund’s complete adjuvant was used for multiple intradermal injections of a male New Zealand White rabbit and included boosting at 3-week intervals. Periodic bleeds from an ear vein were performed following the second boost and allowed to clot at room temperature prior to centrifugation at 2000 × g for 15 min to obtain serum. The aliquots were maintained at −80 °C for long term storage.

Transfection, Metabolic Radiolabeling, and Immunoprecipitation—Transient transfections and cotransfection of H1A cells were performed as previously described, using the Lipopectamine 2000 method (Invitrogen) (18). Changes in the rate of target protein degradation were monitored by pulse-chase metabolic radiolabeling with methionine and immunoprecipitation (15, 18) 72 h post-transfection. In the appropriate experiments, monolayers of semi-confluent cells were subject to preincubation incubated for 1 h at 37 °C in regular growth medium (25) or with medium supplemented with specific inhibitors (lactacystin, 0.025 mM; kifunensine, 0.1 – 0.2 mM; leupeptin, 0.05 mg/ml) as previously reported (18, 25). Metabolic radiolabeling with [35S]methionine (0.150 mCi/100-mm dish; ICN Pharmaceuticals, Inc.) was performed with methionine-free medium (ICN Pharmaceuticals, Inc.) or with medium containing inhibitors (lactacystin, 0.025 mM; kifunensine, 0.1 – 0.2 mM; leupeptin, 0.1 mg/ml; chloroquine, 0.2 mM; brefeldin A, 0.005 mg/ml; staurosporine, 100 mM; or genistein, 0.050 mM) for 10 min, followed by a chase in serum-free Dulbecco’s modified Eagle’s medium (Invitrogen) containing 0.2 mM unlabeled methionine with or without inhibitor, as appropriate. Metabolic incorporation and detection of radiolabeled phosphate into cellular proteins, plus digestion with potato acid phosphatase (Sigma), was performed as previously described. At selected intervals, immunoprecipitates were generated from buffered Nonidet P–40 (Nonidet P–40) detergent (Sigma)-generated cell lysates and medium as previously described (27). Following separation by SDS-PAGE, radiolabeled proteins were detected by fluorography and quantified by densitometric scanning using the National Institutes of Health Image Program or by phosphorimaging analysis (18).
**Human ERManI Checkpoint Control**

**Immunoblotting**—Approximately 48–72 h post-transfection, 5 × 10^6 cells were lysed in 1 ml of buffered Nonidet P-40 detergent (Sigma). Equivalent aliquots were resolved by SDS-PAGE and transferred to nitrocellulose prior to blotting with specific antisera. The signals were detected with the recommended ECL Western blotting reagents (Amersham Biosciences) as previously described (14, 15, 18).

**Deletion of the hERManI Amino-terminal Cytoplasmic Tail**—Programs TMpred from the ExPASy website (us.expasy.org/tools) were used for the identification of amino acids that comprise the amino-terminal cytoplasmic tail of hERManI (accession number NM_007230). PCR was used to modify the hERManI cDNA (accession number NM_007230) to encode a recombinant protein in which the entire amino-terminal cytoplasmic tail was deleted. Priming from nucleotides 234 to 251 was performed with primer HindIIIΔT. (5′-CGAAGCTTCA-GCGAATATGATTCTC) that contained a HindIII site at the 5′ end. Primer ERMITAGRI (5′-GGAATTCCTCCACTTAGGCAAGGGTCC) was used for priming from nucleotides 2106 to 2087 and contained an EcoRI site at the 3′-end. The 1.9-kb PCR fragment was purified and subcloned into pCRII (Invitrogen) for sequence confirmation and then subcloned into the mammalian expression vector pCDNA3.1Zeo+ (Invitrogen) to generate phERManI(NT)/pCDNA3.1Zeo+. The methionine residue originally located at position 49 in wild type hERManI (accession number NM_007230) was used as the new start codon for expression of the recombinant “no tail” protein, hERManI(NT). Positive bacterial transformants were identified by restriction enzyme digestion. Expression of the encoded protein in mammalian cells was confirmed by immunoprecipitation and Western blotting of the mammalian cell extracts following transfection (14, 15, 18).

**Design of Fluorescent Fusion Proteins**—For the expression of fluorescent fusion proteins that contain the amino-terminal cytoplasmic tail, transmembrane domain, and a significant portion of the luminal stem of hERManI (accession number NM_007230), a 500-bp EcoRI (5′-end)-BamHI (nucleotide 569) fragment was purified from DNA construct hERManI/pCDNA3.1Zeo+ (18) and then subcloned into the corresponding sites in pEGFP-N1 and pDsRed2-N1 (BD Biosciences, Palo Alto, CA) to generate plasmids hERManI(1–158)/pEGFP-N1 and hERManI(1–158)/pDsRED2-N1, respectively. To specifically eliminate the cytoplasmic tail, a 350-bp HindIII-BamHI fragment purified from plasmid hERManI/pCDNA3.1Zeo+ (18) was subcloned into the HindIII-BamHI site of pEGFP-N1 or pDsRed2-N1 to generate constructs hERManI(49–158)/EGFP-N1 and phERManI(49–158)/DsRED2-N1, respectively. Positive bacterial transformants were identified by restriction enzyme digestion, and the encoded recombinant proteins were detected by immunoblotting with antibodies against GFP, RFP (red fluorescent protein) (Invitrogen), and hERManI after transfection into mammalian cells.

**Indirect Immunofluorescence and Confocal Microscopy**—24 h-post transfection, the cells were grown on coverslips in 6-well plates for an additional 24 h, then washed in phosphate-buffered saline, air-dried at room temperature, and fixed in methanol for 5 min. For indirect immunofluorescent staining, fixed cells were incubated in phosphate-buffered saline blocking solution containing 1% bovine serum albumin and 0.5% Tween 20 detergent at room temperature for 1 h and then subjected to the primary antibody reactions at 4 °C overnight using a goat polyclonal antibody against lysosomal-associated membrane protein 1 (Lamp1) (1:200 dilution) and the appropriate rabbit polyclonal ERManI antibody (1:200 dilution). Secondary antibody reactions were performed at room temperature, for 1 h, using anti-goat or anti-rabbit IgG conjugated with fluorescein isothiocyanate or Texas Red. For high resolution studies, after mounting in GEL/MOUNT TM (Biomeda Corp., Foster City, CA), fluorescent images were obtained using a Zeiss LSM 510 confocal microscope system and then analyzed with LSM 510 software and Adobe Photoshop v7.

**RESULTS**

**Rapid Intracellular Turnover of Transfected Recombinant Human ER Mannosidase I**—Modification of asparagine-linked oligosaccharides by ER mannosidase I is the rate-limiting step for GERAD in H1A cells (14, 15, 28). Moreover, its experimentally elevated concentration can inappropriately target newly synthesized wild type glycoproteins into GERAD (18). Our immediate objective was to test the prediction that the human homolog is subject to tight intracellular regulation. According to ECL Western blotting of cell lysates, the transiently expressed recombinant human ER mannosidase I was absent from mock transfected cells but detected as a single immunoreactive 79-kDa band following transient expression (Fig. 2A). A combination of metabolic pulse-chase radiolabeling and immunoprecipitation allowed us to monitor the fate of the protein following a 10-min pulse with [35S]methionine. The entire radiolabeled cohort rapidly disappeared from cells (T1/2 = 20 min) and was absent from the medium (Fig. 2, B and C), indicating that its loss was not caused by secretion.

**Involvement of Lysosomal Hydrolases**—Because immunoreactive recombinant human ER mannosidase I was not detected in the insoluble fraction of the Nonidet P-40 cell lysates (data not shown), we concluded that intracellular degradation, rather than insolvability, was responsible for our observation. In mammalian cells, 26 S proteasomes and lysosomes are the two major systems by which proteins are degraded (29). To determine whether either might be responsible for the rapid turnover of transfected human recombinant ER mannosidase I, metabolic pulse-chase radiolabeling and immunoprecipitation were repeated in cells that had been incubated with specific inhibitors of either system. Incubation with lactacystin (0.025–0.1 mM), an irreversible inhibitor of multicatalytic proteasomes (30), had only a minor effect on the disappearance of radiolabeled human ER mannosidase I (Fig. 3A, Lc7) as compared with control (Fig. 3A, Co) during a 120-min chase. Elevated concentrations of the inhibitor did not prove to be more effective (data not shown). In contrast, the entire cohort was detectable following the same period in which the medium was supplemented with chloroquine (Fig. 3A, Clq), a lysosomotrophic amine capable of raising the pH of acidic intracellular compartments. To verify this observation, the cells were incubated in medium containing ammonium chloride, another lysosomotrophic amine (31). Although the treatment exerted a stabi-
lizing effect (data not shown), the apparent cytotoxicity precluded any accurate interpretation of the results. Therefore, incubation with medium supplemented with the lysosomal protease inhibitor leupeptin (32) was performed as an alternative method to test our hypothesis. Under these conditions, intracellular turnover of pulse-radiolabeled recombinant human ER mannosidase I was substantially inhibited (Fig. 3B).

To determine whether the observations reflected a cellular response to the elevation of total ER mannosidase I activity, the fate of the recombinant protein was monitored in transfected cells incubated with kifunensine, a general membrane-permeable inhibitor of α-mannosidase activity (33). However, the manipulation exerted no apparent stabilizing effect (Fig. 3B), Co, normal medium; Lct, medium with the addition of lactacystin; Clq, medium with the addition of chloroquine; Kif, medium with the addition of kifunensine ("Experimental Procedures"). B, same as above, but with the addition of leupeptin (Leup) ("Experimental Procedures"). Duration of the metabolic pulse in B was 10 min.

**FIGURE 2.** Intracellular turnover of transfected (transf.) recombinant human ER mannosidase I. A, detection by ECL Western blotting of transiently expressed recombinant human ER mannosidase I. B, pulse-chase metabolic radiolabeling with [35S]Met and selective immunoprecipitation of recombinant human ER mannosidase I. C, results from B are depicted as a graph. All of the experiments were initiated 48–72 h post-transfection. Specific antiserum ("Experimental Procedures") resulted in the detection of the recombinant protein in Nonidet P-40 cell lysates (cells) and tissue culture medium (media). Duration of the metabolic pulse in B was 10 min.

**FIGURE 3.** Pharmacologic inhibition of recombinant human ER mannosidase I turnover. A, pulse-chase metabolic radiolabeling with [35S]Met and selective immunoprecipitation of recombinant human ER mannosidase I. Co, normal medium; Lct, medium with the addition of lactacystin; Clq, medium with the addition of chloroquine; Kif, medium with the addition of kifunensine ("Experimental Procedures"). B, same as above, but with the addition of leupeptin (Leup) ("Experimental Procedures"). Duration of the metabolic pulse in B was 10 min.

**Physiologic Relevance of ER Mannosidase I Destabilization**—Because short-lived proteins undergo intracellular depletion in response to the inhibition of protein synthesis, the intracellular concentration of endogenous mouse ER mannosidase I was examined following a 1-h incubation in regular growth medium supplemented with cycloheximide. ECL Western blotting of Nonidet P-40 cell lysates demonstrated that the inhibition of translation resulted in a reduction in the level of the endogenous mouse homolog (Fig. 5, compare A and B). The absence of any demonstrable change in the intracellular concentration of the long-lived ER resident molecular chaperone grp78/BiP (34), as compared with control (Fig. 5A, lower panel), provided confirmatory evidence for the short intracellular half-life of ER mannosidase I under normal conditions.
Intracellular depletion of ER mannosidase I and concomitant inhibition of GERAD in response to a 1-h incubation in medium containing cycloheximide. A, detection of endogenous mERManI and grp78/BiP by ECL Western blotting from Nonidet P-40 cell lysates under control conditions (Co) or following incubation with cycloheximide (Chx). Protein G used without antiserum (pG). B, pulse-chase metabolic radiolabeling with [35S]Met and immunoprecipitation of α1-antitrypsin variant null(Hong Kong) (NHK) in cell line H1A/N13 ("Experimental Procedures") under control conditions (Co) or following treatment with cycloheximide (Chx) or kifunensine (Kif). Duration of the metabolic pulse in B was 10 min.
ments the rate of intracellular turnover was significantly diminished as compared with control in cells incubated with medium containing staurosporine (Fig. 6D), a membrane-permeable inhibitor of general Ser/Thr kinase activity. In contrast, genistein (Fig. 6D), a general protein-tyrosine kinase inhibitor (38), had no apparent effect. Taken together, the data argue against the notion that human ER mannosidase I is merely labile.

**Contribution by the Amino-terminal Cytosolic Tail**—One interpretation of the aforementioned data is that human ER mannosidase I is subject to regulated proteolysis. The modular architecture of the glycosidase was exploited to identify a domain through which its down-regulation might be mediated. The prediction was that removal of a candidate regulatory domain would ablate rapid intracellular turnover. Therefore, the fate of the protein was monitored following genetically engineered removal of the amino-terminal cytoplasmic tail. Removal of the tail does not influence the remaining structure of the molecule, including the original transmembrane domain, noncleavable ER sorting signal, and proposed stem and catalytic domains (Fig. 7A). The fates of wild type (WT) and tail-less (NT) human ER mannosidase I were monitored by metabolic pulse-chase radiolabeling following transient transfection. Both proteins were synthesized as single radiolabeled bands, although of slightly different molecular weights, as predicted (Fig. 7B). Importantly, its absence did not diminish the efficiency of protein translation. As shown in Fig. 7B, the radiolabeled wild type molecules were rapidly degraded during a 1-h chase, whereas the tail-less counterpart remained stable during this period. Stabilization of the tail-less molecule implied that it had not misfolded, otherwise the molecules would have been subject to intracellular destruction by ERAD.

In a subsequent set of experiments, ECL Western blotting demonstrated that recombinant NT was phosphorylated under steady state conditions (Fig. 7C). The observation precluded any conclusion of the involvement of the tail in targeting ER mannosidase I to lysosomes. Therefore, to further investigate the involvement of the tail, fluorescent fusion proteins were generated in which the luminal stem of human ER mannosidase I was fused in-frame to GFP (Fig. 8A). The stem was chosen as the site for fusion because the putative signal might
extend into the transmembrane domain. Also, the absence of distinct structural features in the stem would prevent random misfolding of the chimeric molecules.

Our initial experiments demonstrated that incubation with chloroquine disrupted the intracellular architecture of H1A cells (not shown), precluding our original strategy to test for colocalization with lysosomes. Therefore, the experimental design was performed under steady-state conditions. A considerable fraction of recombinant hERMI (1–158)-GFP colocalized with the endogenous lysosomal marker, Lamp1 (Fig. 8A, top set of panels). To substantiate the involvement of the ER mannosidase I tail, the encoding nucleotides were selectively removed (Fig. 8B). Consistent with our hypothesis, the manipulation ablated any detectable colocalization with Lamp1 (Fig. 8B, middle panels). Importantly, the tail-less chimeric molecule had apparently sequestered within cells (Fig. 8B, middle panels). To identify the apparent site of accumulation, GFP was replaced with red fluorescent protein (RFP) (Fig. 8A) to allow for analyses following coexpression with the cyan-colored RhoB-CFP. Colocalization of the two molecules (Fig. 8B, lower panels) indicated that removal of the tail had led to accumulation of the recombinant fusion proteins in an expanded endosomal compartment (39). In no set of experiments were any of the fluorescent fusion proteins detected at the cell surface (not shown). These findings imply that sequestration in endosomes likely represents mistargeting of the tail-less fusion protein rather than identification of a compartment through which the molecules are normally transported to lysosomes.

DISCUSSION

Successful gene expression is rooted in the correct conformational maturation of encoded proteins and selective removal of those molecules unable to attain this structural milestone. The earliest events by which cells manage the numerous proteins translocated into the ER is central to the branch point composed of protein folding and degradation. Considering the stochastic role played by the low basal ER mannosidase I concentration and its potential contribution to a post-translational checkpoint, we asked whether the molecule is subject to regulated proteolysis.

In our present study, an analysis of the endogenous mouse ortholog was incorporated into the experimental design to ensure that no observations were artifactual. Rapid intracellular turnover of the transfected molecules confirmed the existence of a short intracellular half-life. The conclusion was strengthened by the capacity of cycloheximide treatment to deplete cells of ER mannosidase I. The physiological relevance of ER mannosidase I destabilization was verified by the short half-life of the endogenous mouse ER mannosidase I, plus its depletion in response to cycloheximide treatment and ability to mimic the effect of kifunensine on the fate of a GERAD substrate. Importantly, our findings begin to address the long-standing question as to how cycloheximide is capable, at least in some situations, to stabilize multiple GERAD substrates (40). However, it should be noted that cycloheximide is capable of influencing numerous intracellular systems. Therefore, it is premature to propose its use as a diagnostic tool for the identification of GERAD clients.

Pharmacological inhibition studies, plus intracellular colocalization of GFP fusion proteins with Lamp1, provided separate lines of evidence that proteolytic regulation of human ER mannosidase I involves lysosomal proteases. The simplest explanation for using lysosomes, rather than proteasomes, to down-regulate ER mannosidase I is to avoid the potential complications that might arise from controlling the glycosidase concentration by the system for which it is intended to function. Involvement of the amino-terminal cytoplasmic tail is reasonable, especially when one considers the difference in length between that of the human and yeast orthologs (9, 41).

Prior to this study, the underlying clockwork mechanics by which the critical glycan-based signal determinant is generated had remained poorly defined (2, 18). Our observations challenge the prior notion that the poor catalytic activity of ER mannosidase I (9, 10) functions as the sole mechanism by which the rate at which glycoproteins are targeted for GERAD. Of greater importance, the data imply that long-lived cellular factors, such as alternative mannosidases, are unable to compensate for the role played by ER mannosidase I in GERAD. After the initiation of our study, Nagata and co-workers (21) reported the rapid turnover of recombinant human ER mannosidase I by a nonproteasomal pathway when transiently expressed in a cul-

Human ERManI Checkpoint Control
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tured human embryonic kidney cell line. However, no explanation about the possible physiologic relevance of their observation was provided.

Based on our findings, we conclude that ER mannosidase I is subject to regulated proteolysis as a means to govern the performance-based measurement of inefficient glycoprotein conformational maturation. The intracellular ER mannosidase I concentration represents a quantitative trait that underlies the critical decision of how long a nonnative glycoprotein is tolerated, although it is difficult to reconcile how the rapid intracellular ER mannosidase I instability remains elusive. The same can be said concerning coevolution of cell cycle checkpoint regulation and ER quality control.

The role by which phosphorylation might contribute to ER mannosidase I instability remains elusive. The same can be said for the stabilizing effect of brefeldin A. Presently, we do not know how the glycosidase is transported to lysosomes. Also, because the proteolytic down-regulation of human ER mannosidase I is operative under basal conditions, it is not yet known whether some natural cellular strategy might exist to adjust the glycosidase concentration under conditions of ER stress, possibly at a post-translational level. Moreover, despite our conclusions it should be noted that Lederkremer and Glickman (45) have proposed a model in which vesicle recycling rather than strict ER retention contributes to the "timed" degradation (46) of secretion-competent glycoproteins. In that model nonnative protein structure diverts glycoproteins into a specific targeting pathway, and a window of opportunity in which glycoproteins are exposed to ER mannosidase I represents the emergent clock. Additional investigation of the model is warranted, although it is difficult to reconcile how the rapid intracellular degradation of the glycosidase might contribute to this process.

Finally, it should be mentioned that the secretion of structurally aberrant proteins from cultured mammalian cells has been reported (47), bringing into question the actual existence of quality control. Our findings indicate that the system does, in fact, exist and plays a vital role in the selection of substrates for GERAD. At least four explanations might explain the unexpected phenomenon in which structurally aberrant proteins are secreted. These are: (i) the failure of heterologous systems to adequately monitor foreign proteins, (ii) the saturation of endogenous folding, retention, and/or degradation systems, (iii) aberrant cell adaptation in response to prolonged cell culture that results in the loss of retention and/or ERAD elements, and/or (iv) a previously unanticipated aspect of the overall system.

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