Molecular Pathogenesis of Genetic and Inherited Diseases

Mutant Fibrinogen Cleared from the Endoplasmic Reticulum via Endoplasmic Reticulum-Associated Protein Degradation and Autophagy

An Explanation for Liver Disease

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The endoplasmic reticulum (ER) quality control processes recognize and remove aberrant proteins from the secretory pathway. Several variants of the plasma protein fibrinogen are recognized as aberrant and degraded by ER-associated protein degradation (ERAD), thus leading to hypofibrinogenemia. A subset of patients with hypofibrinogenemia exhibit hepatic ER accumulation of the variant fibrinogens and develop liver cirrhosis. One such variant named Aguadilla has a substitution of Arg375 to Trp in the γ -chain. To understand the cellular mechanisms behind clearance of the aberrant Aguadilla γ -chain, we expressed the mutant γ D domain in yeast and found that it was cleared from the ER via ERAD. In addition, we discovered that when ERAD was saturated, aggregated Aguadilla γ D accumulated within the ER while a soluble form of the polypeptide transited the secretory pathway to the *trans*-Golgi network where it was targeted to the vacuole for degradation. Examination of Aguadilla γ D in an autophagy-deficient yeast strain showed stabilization of the aggregated ER form, indicating that these aggregates are normally cleared from the ER via the autophagic pathway. These findings have clinical relevance in the understanding of and treatment for ER storage diseases. (*Am J Pathol* 2006, 168:1299–1308; DOI: 10.2353/ajpath.2006.051097)

Fibrinogen, a large plasma protein synthesized in hepatocytes, plays a critical role in blood coagulation. Mature circulating fibrinogen is a symmetric dimeric molecule in which each half consists of three polypeptide chains: A α , B β , and γ . The two halves of the molecule associate into a trinodal D-E-D structure with the N termini of all six chains in the central E domain, the C termini of B β and γ forming the two globular D domains, and the C termini of the A α -chains forming a less compact structure associated with the E domain.^{1,2} Assembly of all six chains into the fibrinogen molecule occurs within the hepatocyte endoplasmic reticulum.^{3–5} Individual unassembled fibrinogen chains are retained within the endoplasmic reticulum (ER) and ultimately degraded in a proteasome-dependent manner, classifying them as substrates of ER-associated protein degradation (ERAD).^{5,6}

The identification of the genetic defects associated with low levels of circulating fibrinogen (hypofibrinogenemia) has shown that mutations within any of the three chains can lead to misfolding of that chain, recognition of the aberrant polypeptide by ER quality control, and subsequent degradation.⁷ However, studies of individuals carrying various mutant forms of fibrinogen chains have shown that a subset of those individuals with hypofibrinogenemia also exhibit hepatic ER accumulation of fibrinogen that appears to lead to liver disease.^{8–10} For example, we previously described one family harboring a novel γ -chain mutation, Arg375Trp, termed fibrinogen Aguadilla, in which all carriers present with hypofibrinogenemia, but only a subset have liver inclusions.¹⁰ Biopsies from diseased livers showed aggregates of fibrinogen within the ER lumen of hepatocytes.¹⁰ This disease pathology, ie, both the loss-of-function hypofibrinogen-

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emia and the gain-of-function liver disease, is similar to α -1-antitrypsin deficiency, another ER storage disease.

Individuals who are homozygous for the Z variant of the serum protein α -1-antitrypsin (α -1-protease inhibitor, A1Pi) exhibit low levels of A1Pi and the resulting loss-of-function disease pathology of early onset emphysema.¹¹ In addition, 12 to 15% of A1PiZ homozygous juveniles also display the gain-of-function disease pathology of liver cirrhosis,^{12–15} and studies of adults indicate a risk of cirrhosis in 30 to 50% of homozygous individuals.¹⁶ It has been previously demonstrated, in both mammalian and yeast cell systems, that the intracellular fate of A1PiZ is ERAD.^{17,18} However, when this pathway is saturated such that it is insufficient to rid the ER of misfolded A1PiZ, aggregates accumulate within the ER.¹⁹ Interestingly, the clearance of these A1PiZ aggregates from the ER is dependent on the autophagic pathway to the vacuole.¹⁹ These findings correlate with the observation that there is an increased number of autophagosomes in hepatocytes of A1PiZ homozygous individuals with liver disease.²⁰ Together, these data suggest that liver disease associated with α -1-antitrypsin deficiency may be due to different inherited efficiencies in ER quality control or possibly to a second mutation within the ERAD or autophagy pathways.

Because of the similarity between the pathology of α -1-antitrypsin deficiency and hypofibrinogenemia-associated liver disease, we reasoned that the fibrinogen Aguadilla Arg γ 375Trp variant would have an intracellular fate similar to that of A1PiZ. Thus, to investigate the mechanism involved in the fibrinogen Aguadilla-related ER storage disease, we decided to express the fibrinogen γ D domain with the Arg γ 375Trp mutation in our yeast model system. The rationale for the use of the γ D domain comes from previous studies showing that when expressed in *Pichia pastoris*, the normal γ D domain is correctly folded into a stable configuration with both its calcium binding site and polymerization pocket intact.²¹ Should the Aguadilla mutation affect folding of the fibrinogen γ D domain, we anticipate that the quality control mechanisms that contribute to clearance of the aberrant polypeptide may be identified in our yeast system. Knowledge of these quality control mechanisms could begin to answer questions regarding the molecular mechanisms driving the pathology of hypofibrinogenemia and liver disease.

Materials and Methods

Strains, Plasmids, Media, and Antisera

Electrocompetent *Escherichia coli* strain HB101 (F^- , *mcrB*, *mrr*, *hsdS20*(r_B^- , m_B^-), *recA13*, *supE44*, *ara14*, *galK2*, *lacY1*, *proA2*, *rpsL20*(Sm^r), *xyl5*, λ^- , and *mtl*) was purchased from Invitrogen Corp. (Carlsbad, CA). The *P. pastoris* strain used in this study was GS115/his4 (Invitrogen). The *Saccharomyces cerevisiae* strains used were wild type²²: BY4742/*MAT α* , *his3 Δ 1*, *leu2 Δ 0*, *lys Δ 0*, and *ura3 Δ 0*; BY4742: *pep4 Δ 22*/*MAT α* , *his3 Δ 1*, *leu2 Δ 0*, *lys Δ 0*, *ura3 Δ 0*, and *YPL154c::KANMX* (Invitrogen);

BY4742: *atg14 Δ 19*/*MAT α* , *his3 Δ 1*, *leu2 Δ 0*, *lys Δ 0*, *ura3 Δ 0*, and *YBR128c::KANMX4*; the proteasome mutant²³: *pre1-1*, *pre2-2*/*MAT α* , *leu2-3*, 112, *his3-11*, 15, *ura3(Δ 5)*, *GAL+*, *can(s)*, *pre1-1*, and *pre2-2*; and the wild-type isogenic parent²³: WCG4/*MAT α* , *leu2-3*, 112, *his3-11*, 15, *ura3(Δ 5)*, and *GAL+* (kindly provided by Dieter Wolf, University of Stuttgart, Germany).

P. pastoris yeast cells were cultured in synthetic media containing 1.34% yeast nitrogen base without amino acids and ammonium sulfate, 2% peptone, 1% yeast extract, 100 mmol/L potassium phosphate, pH 6.0, 400 ng/ml biotin, and 1% glycerol as a carbon source or with 1% methanol to induce expression of γ D fibrinogen. *S. cerevisiae* yeast cells were cultured in synthetic media containing 0.67% yeast nitrogen base with amino acids, 0.5% casamino acids, and 2% dextrose as a carbon source or with 2% galactose to induce expression of γ D fibrinogen. Antibodies used included rabbit anti-human fibrinogen (DakoCytomation California Inc., Carpinteria, CA) and horseradish peroxidase-conjugated goat anti-rabbit (U.S. Biochemical Co., Cleveland, OH), monoclonal mouse anti-yeast phosphoglycerate kinase (Invitrogen), rabbit anti-Kar2p²⁴ (generously provided by Jeffrey Brodsky, University of Pittsburgh, Pittsburgh, PA), and sheep anti-mouse IgG horseradish peroxidase-conjugated antibody (GE Health Care Bio-Sciences, Little Chalfont, UK).

Construction of Expression Vectors

The integrative expression vector, pPIC9 (Invitrogen), carrying the coding regions spanning amino acid residues 143 to 411 of the human fibrinogen γ -gene²⁵ (hereafter referred to as the fibrinogen γ D domain) was kindly supplied by Dr. Tatiana Ugarova (Cleveland Clinic Foundation, Cleveland, OH). The fragment encoding the fibrinogen γ D domain was cloned in frame with the yeast α -factor secretion signal and propeptide and driven by an alcohol oxidase 1 methanol-inducible promoter. The fibrinogen Aguadilla mutation, Arg γ 375Trp,¹⁰ was inserted using mutagenic primers (available on request) with a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), and the mutation was verified using automated sequencing. Using polymerase chain reaction and primers that introduced unique restriction sites, the α -factor pre-pro and γ -sequences were subcloned into an *S. cerevisiae* expression vector with a regulatable promoter²⁶: either the inducible *GAL1* promoter p426Gal1 (2 μ , Amp^r, and *URA3*; American Type Culture Collection, Manassas, VA) or the repressible *MET25* promoter p426MET25 (2 μ , Amp^r, and *URA3*; American Type Culture Collection). Correct cloning was verified by restriction digest and automated sequencing.

Transformations and Plasmid Preparation

The Cell-Porator *E. coli* Pulser (Invitrogen) was used to electroporate competent *E. coli*. Plasmids were isolated from bacterial transformants using the Quantum Prep Plasmid Miniprep kit (Bio-Rad, Hercules, CA). *P. pastoris*

transformations were performed using the spheroplasting protocol within the Pichia Expression kit M instruction manual (Invitrogen), and transformants were selected in medium lacking histidine. Southern blot was used to choose transformants with only a single copy²⁷ of the integrative pPIC9 expression vector. *S. cerevisiae* transformations were performed using a standard lithium acetate procedure,²⁸ and transformants were isolated after growth in selective medium containing 2% dextrose.

ERAD Assays

The colony-blot immunoassays for both *S. cerevisiae* and *P. pastoris* were a modification of a previously described procedure.¹⁹ In brief, exponentially growing yeast were resuspended to a final optical density (OD; at 600 nm) of 0.001 OD/ μ l to control for cell number. A 3- μ l aliquot of these yeast were spotted onto a nitrocellulose disk overlaid on medium containing 1% methanol to induce expression in *P. pastoris* of the γ D off the AOX1 promoter, medium containing 2% galactose to induce expression in *S. cerevisiae* of the γ D off the *GAL1* promoter, or synthetic medium lacking methionine to induce expression in *S. cerevisiae* of the γ D off the *MET25* promoter. After incubation at 30°C for 18 hours, cells were lysed with solution 1 (0.2 mol/L NaOH, 0.1% sodium dodecyl sulfate (SDS), and 0.05% 2-mercaptoethanol) for 1 hour at room temperature, and blots were then assayed as previously described.²⁹ Immunoreactive proteins were detected by developing the blots with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and Super RX X-ray film (Fuji, Tokyo, Japan). The density of immunoreactive protein at each colony spot was quantified using Molecular Analyst (Bio-Rad).

For pulse-chase protein radiolabeling to assay for γ D degradation in *P. pastoris*, cultures were grown at 30°C in medium containing 1% methanol to induce expression for 24 hours before analysis. Using a protocol based on those previously described,^{30,31} cells were resuspended at 4 OD/ml in medium lacking histidine, cysteine, and methionine. They were incubated at 30°C for 45 minutes, then pulsed with 25 μ Ci/OD EasyTag Express Protein Labeling Mix (PerkinElmer Inc., Boston, MA) for 10 minutes, and chased with 1 mg/ml cold cysteine and methionine. Aliquots were removed at the desired time points. Cell lysates were prepared and immunoprecipitated with rabbit polyclonal antibody to human fibrinogen. The purified proteins were separated by 10% reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography, and signal intensities were quantified using Molecular Analyst (Bio-Rad).

The cycloheximide chase analyses of *S. cerevisiae* were a modification of a previously described procedure.¹⁹ In brief, overnight cultures were incubated in medium containing 2% galactose for 4 to 8 hours at 30°C. To control for cell number, cultures were then resuspended to a final OD₆₀₀ of 2 OD/ml in medium containing 2% dextrose plus 200 μ g/ml cycloheximide. They were incubated at 30°C with shaking, 500- μ l aliquots were collected, and the cells were harvested by centrifugation

at the indicated time points. At each time point, the medium was reserved and represented the secreted material. The cell pellets were resuspended in 50 μ l of lysis buffer (160 mmol/L Tris, pH 6.8, 4% SDS, 0.2% bromophenol blue, 200 mmol/L dithiothreitol [DTT], and 20% glycerol) and incubated at 95°C for 2 minutes, and 0.5-mm acid-washed glass beads were added. The cells were disrupted via four sequential 60-second bursts on a Vortex mixer at the highest setting followed by cooling on ice for 60 seconds. Finally, an additional 50- μ l aliquot of lysis buffer and the reserved media were added, resulting in 0.0017 OD/ μ l final concentration. Then, 20- μ l aliquots of lysate/media were combined with 10 μ l of sample buffer (0.125 mol/L Tris, pH 6.8, 4% SDS, 0.004% bromophenol blue, 10% 2-mercaptoethanol, and 20% glycerol), heated to 95°C for 10 minutes, and resolved by 10% reducing SDS-PAGE. The γ D domain was detected by immunoblot analysis, and results were quantified as described above.

Protein Characterization Studies and Sequencing

P. pastoris cultures were grown in medium containing 1% methanol for 72 hours at 30°C. Cell pellets were lysed by vortexing with acid-washed glass beads as described above but in 50 mmol/L Tris-HCl, pH 7.4, and 1 mmol/L EDTA, washed twice with 1% Triton X-100 and then three times with water. The remaining pellet was solubilized in 8 mol/L urea, 100 mmol/L Tris-HCl, pH 8.0, and 15 mmol/L DTT and reduced for 16 hours at room temperature.

The high and low molecular weight γ D species were separated by reverse phase high performance liquid chromatography (HPLC) on a 5- μ m Jupiter C4 column (Phenomenex, Torrance, CA) with a 0.05% trifluoroacetic acid/acetonitrile gradient similar to that used for reduced human fibrinogen chains.³² Peaks were collected and assayed at the Protein Microchemistry Facility at the University of Otago, New Zealand for N-terminal protein sequencing using established methods.³³ A 20- μ l sample of each peak crest was also analyzed by electrospray ionization mass spectrometry (ESI MS) on a VG Platform II quadrupole analyzer (Micromass, Manchester, UK) as previously described.³²

For peptide mapping, HPLC peaks were dried, redissolved in 50 μ l of 50 mmol/L ammonium carbonate and digested with 2 μ g of trypsin for 16 hours at 37°C. After being dried under vacuum with P₂O₅ and redissolved in 50 μ l of 50% acetonitrile and 0.1% formic acid, 20 μ l of each digest was analyzed by ESI MS.

Cell Fractionation

S. cerevisiae cultures were grown in medium containing 2% galactose for 40 hours at 30°C to induce expression of γ D. Yeast were pelleted, and the medium was reserved and concentrated fourfold for use as the extracellular, secreted fraction. Spheroplasts were formed by incubating cells with 10 mg/ml lyticase in 1.2 mol/L sor-

bitol and 0.1 mol/L K_2HPO_4 , pH 7.2, and spheroplasts and the periplasm fraction were separated by centrifugation through a cushion of 0.8 mol/L sucrose, 1.5% Ficoll, and 20 mmol/L HEPES, pH 7.4, at $4000 \times g$ for 10 minutes at 4°C. After the spheroplasts were washed with 1.2 mol/L sorbitol and 0.1 mol/L K_2HPO_4 , pH 7.2, they were resuspended in ice-cold Spheroplast lysis buffer (0.1 mol/L sorbitol, 50 mmol/L KOAc, 2 mmol/L EDTA, 20 mmol/L HEPES, pH 7.4, 1 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride) and lysed by 10 strokes with a dounce homogenizer on ice. Resulting cell lysates were layered over a cushion of 1 mol/L sucrose, 50 mmol/L potassium acetate, 20 mmol/L HEPES, pH 7.4, and 1 mmol/L DTT and subjected to $6500 \times g$ centrifugation for 10 minutes at 4°C. The top fraction, above the interface, was collected and subjected to further centrifugation at $22,000 \times g$ for 10 minutes at 4°C. The resulting pellet was the microsome fraction, and the supernatant/cytosol was further cleared of small vesicles by centrifugation at $100,000 \times g$ for 1 hour at 4°C. Protease protection assays were performed on the microsome fraction by exposing washed microsomes either to 0.4 mg/ml trypsin in buffer 88 (20 mmol/L HEPES, pH 6.8, 150 mmol/L KOAc, 250 mmol/L sorbitol, and 5 mmol/L MgOAc) or to 0.4 mg/ml trypsin and 2% Triton X-100 in buffer 88 for 30 minutes on ice. Specific proteins were then visualized by immunoblot analysis.

Sucrose Density Gradient Analysis

Microsomes were isolated as described above from *S. cerevisiae* producing γ D during 40 hours of induction. The microsomes were lysed in buffer 88 that had been supplemented with 0.5% Triton X-100 and complete protease inhibitor cocktail (Sigma, St. Louis, MO) by incubation on ice for 2 hours, with brief vortex mixing every 30 minutes. The total protein content of the lysates was determined using Coomassie Plus Protein Assay (Pierce). An aliquot of 1 mg of total protein from each lysate was loaded onto a 5 to 60% sucrose gradient^{34,35} and centrifuged in a Beckman (Beckman Coulter, Inc., Fullerton, CA) SW50 at $145,000 \times g$ for 20 hours at 4°C. Fractions of 250 μ l were collected, and proteins were concentrated by precipitation in 10% trichloroacetic acid. Specific proteins were visualized by immunoblot analysis.

Endoglycosidase H Analysis

Microsomes were prepared as described above and lysed on ice for 30 minutes in denaturing buffer (50 mmol/L sodium acetate, pH 5.2, 0.5% 2-mercaptoethanol, and 0.5% SDS). Samples were heated to 95°C for 3 minutes and then centrifuged $14,000 \times g$ for 5 minutes at 4°C to remove membranes. An equal volume of reaction buffer (50 mmol/L sodium acetate, pH 5.2, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 0.5% NP-40) was added to the supernatant, and the sample was split in two. One sample received 0.05 units of endoglycosidase H (Roche Diagnostics, Indianapolis, IN), both samples

were incubated at 37°C for 1 hour, and proteins were visualized by immunoblot analysis.

Results

Aguadilla Variant of γ -Fibrinogen Selectively Degraded in Yeast

The ~30-kD C-terminal domain of the γ -chain of human fibrinogen (Val143 through Val411) cloned in-frame behind the yeast α -factor pre-pro sequence was used for these studies because this γ D domain had previously been shown to correctly fold and function.^{21,25} Furthermore, the site of the Aguadilla mutation is within the γ D domain, and the Arg375Trp mutation is believed to alter folding such that it would be recognized by ER quality control.¹⁰ Site-directed mutagenesis was used to generate the fibrinogen Aguadilla mutation. The wild-type and Aguadilla γ D were subcloned into the *P. pastoris* shuttle vector as well as the appropriate shuttle vectors for expression in *S. cerevisiae*.

To examine the fates of wild-type and Aguadilla γ D, both were expressed in *P. pastoris* and *S. cerevisiae*, and expression was examined using a colony-blot immunoassay that allows for quantification of the steady-state level of γ D using an antibody to fibrinogen. In both *P. pastoris* and *S. cerevisiae*, the steady-state level of Aguadilla γ D was less than that seen for the wild-type γ D (Figure 1A), suggesting degradation of the mutant that mimics the phenotype of individuals who carry the Aguadilla mutation, ie, low levels of fibrinogen.¹⁰

To determine whether the low level of Aguadilla γ D was due to degradation, kinetic studies were undertaken in both yeast systems: pulse-chase protein radiolabeling studies were performed in *P. pastoris*, and cycloheximide chase analyses were performed in *S. cerevisiae* (Figure 1, B and C). In both yeast systems, using either chase protocol, two molecular weight species were observed. Furthermore, the low molecular weight (LMW) form of the wild-type γ D was relatively stable over the chase period, whereas the LMW form of the mutant Aguadilla γ D species was clearly degraded by both *P. pastoris* and *S. cerevisiae*.

The γ D fragment encodes a ~30-kD polypeptide²¹ that correlates well with the LMW species, but the presence of a high molecular weight (HMW) species running at approximately 48 kD on reducing SDS-PAGE was puzzling. To characterize this material, lysates from *P. pastoris* were fractionated by reverse phase HPLC (Figure 2A). Analysis of individual peaks by Western blot showed that the LMW species eluted just ahead of the more hydrophobic HMW form (Figure 2B). Direct ESI MS analysis of peak 1 established a mass of 30,470 D, which was in excellent agreement with the theoretical mass of 30,471 D expected for mature γ D. Repeated analysis of the HMW material was unfortunately less informative. However, an N-terminal protein sequence of APVNTTTE-DET- was established, indicating that the α -factor pre sequence had been cleaved, but the pro sequence was still intact. The anticipated molecular mass of the γ D

Figure 1. Agudilla γ D degraded in *P. pastoris* and *S. cerevisiae*. **A:** Representative colony-blot immunoassays of single-copy transformants of *P. pastoris* and high-expression transformants of *S. cerevisiae*, each expressing wild-type γ D (WT γ) or Agudilla γ D (Ag γ) or containing a vector control (C). **B:** A representative pulse-chase immunoprecipitation of single-copy transformants of *P. pastoris* expressing wild-type γ D (WT γ) or Agudilla γ D (Ag γ) or containing the vector control (C). Protein standards are noted to the right in kilodaltons. A high molecular weight ($\blacktriangleleft$) and low molecular weight ($\blacktriangleright$) form of γ D are indicated. **C:** A representative cycloheximide chase immunoblot of a *S. cerevisiae* wild-type strain expressing either wild-type γ D (WT γ) or Agudilla γ D (Ag γ) at high levels off the p426Gal1 vector. A graph of the means $\pm$ SEM, from at least three experiments is shown with wild-type γ D LMW (solid triangles and solid line), wild-type γ D HMW (open triangles and solid line), Agudilla γ D LMW (solid circles and dashed line), and Agudilla γ D HMW (open circles and dashed line).

domain with an N-terminal pro sequence is 38 kd, and the three N-linked oligosaccharides of the propeptide³⁶ increase the predicted molecular mass to approximately 47 kd, close to the observed value of $\sim$ 48 kd. Furthermore, tryptic peptide mass mapping confirmed the primary structures of the two components (Figure 2C). Maps of the LMW material showed the expected ions arising solely from the γ D domain, including the C-terminal pep-

tide. These ions were also present in the HMW digests, together with unique ions arising from the propeptide sequence.

The absence of a pre sequence indicated that all of the γ D material had entered the rough ER, the site of the signal peptidase, whereas the presence of the pro sequence indicated material that had not progressed to the *trans*-Golgi network (TGN), the site of the KEX₂ protease.^{37,38} We addressed whether the pro sequence was glycosylated by isolating a membrane fraction enriched for ER-derived microsomes and treating the γ D species found within the microsomes with endoglycosidase H, an enzyme that specifically cleaves N-linked carbohydrates that were added in the ER. The results seen in Figure 3 show that the microsomes-enriched membrane fraction contains only the HMW species and furthermore that the HMW γ D is sensitive to endoglycosidase H, indicating the γ D had entered the ER and that the pro sequence had been glycosylated.

These findings allowed a more complete interpretation of the data shown in Figure 1, B and C. The HMW ER species of wild-type γ D with the pro sequence intact appeared to be either slowly degraded or, more likely, in transit to the TGN where pro sequence cleavage occurs. The stable species of wild-type γ D was the LMW form with the pro sequence cleaved, suggesting it was within or beyond the TGN. Studies in both yeast systems demonstrated that the LMW wild-type γ D was efficiently secreted, as shown for *S. cerevisiae* in Figure 3. Conversely, both species (LMW and HMW) of the mutant Agudilla γ D were reduced over time, with no evidence of secreted material (our unpublished data), indicating selective degradation.

Agudilla γ D Degradation Is Proteasome-Dependent

ERAD has been demonstrated to play a role in the degradation of fibrinogen chains that are aberrant or do not properly assemble within the ER.^{3,6,39} Thus, we hypothesized that ERAD would be the mechanism for degradation of the Agudilla γ D domain. The role of ERAD was examined in the *pre1-1*, *pre2-2* strain of *S. cerevisiae*, a proteasome-deficient strain in which ERAD substrates are stabilized.^{18,23} The wild-type and Agudilla γ D cassettes, cloned into a Met25 low-level expression vector, were transformed into the isogenic wild-type and proteasome mutant strains, and the steady-state level was monitored by colony blot immunoassay (Figure 4A). Stabilization of the Agudilla γ D was clearly seen in the *pre1-1*, *pre2-2* proteasome mutant strain, demonstrating proteasome-dependent degradation and thus implicating ERAD.

Degradation of the LMW Agudilla γ D Is Vacuole-Dependent

Our studies in the proteasome mutant illustrate that Agudilla γ D, when expressed at low levels, is cleared from

Figure 2. Structural analysis of intracellular forms of γ D. **A:** Reverse phase HPLC profile of *P. pastoris* lysate solubilized in 8 mol/L urea, 100 mmol/L Tris-HCl, pH 8.0, and 15 mmol/L DTT. **B:** Western blot of peaks 1 and 2 together with a fibrinogen control (lane C); the mass of the γ -chain of fibrinogen is 48 kD. **C:** ESI MS tryptic peptide maps of the LMW γ D species (top spectrum) and HMW γ D species (bottom spectrum). Both maps show the same expected C-terminal QAGDV ion at 489 m/z , indicating normal transcription termination. However, the N-terminal ion of γ D (YVVQIHDITGK; 637 m/z) is missing in the HMW map and replaced by an 837 m/z ion composed of the same sequence together with the C-terminal portion of the propeptide (EAEA YVVQIHDITGK). The 890 m/z ion (EEGVSLK) is unique to the HMW material and is also predicted to originate from the pro peptide.

the cell by ERAD. But at high levels of expression, do overflow pathways handle the excess aberrant Aguadilla polypeptide? As shown in Figure 1C, both the HMW and LMW species of the Aguadilla γ D were degraded over time, with no build-up of the LMW γ D species. Degradation of the LMW species of Aguadilla γ D suggested that the mutant Aguadilla polypeptide was selected for degradation after arriving in the TGN and after having the pro sequence removed. The selection of aberrant polypeptides within the TGN for trafficking to the vacuole and subsequent degradation has been seen when ERAD has been overwhelmed⁴⁰ or when mutant proteins either escape or are not recognized by ER quality control.^{41–45} In a previous study, we found that when overexpressed, soluble A1PiZ was transported through the biosynthetic pathway from the ER to the Golgi and at the TGN was selectively trafficked via the endosome to the vacuole for degradation.¹⁹ Thus, we next asked whether LMW Aguadilla γ D that had arrived in the TGN was recognized as

aberrant and selectively trafficked to the vacuole for degradation.

The role of the vacuole in degrading Aguadilla γ D was examined by cycloheximide chase analysis in a *S. cerevisiae* *pep4Δ* mutant,⁴⁶ a knock-out of vacuolar proteinase A, an enzyme required for posttranslational precursor maturation of a number of vacuolar proteases. Consequently, the *pep4Δ* mutant lacks most vacuolar protease activity, resulting in significant stabilization of most substances delivered to the vacuole. We found that for Aguadilla γ D, the LMW pro sequence cleaved form was stabilized in the *pep4Δ* strain (Figure 4B), demonstrating that the Aguadilla γ D was recognized as aberrant by quality control mechanisms within the TGN and selectively trafficked to the vacuole for degradation. The HMW form of Aguadilla γ D was also stabilized in the *pep4Δ* strain (Figure 4B), suggesting vacuole-dependent degradation of this species as well, and possibly delivery to the vacuole via the autophagic pathway.

Figure 3. HMW γ D harbors a glycosylated pro sequence. A representative immunoblot of fractions prepared from the *S. cerevisiae* wild-type strain expressing high levels of wild-type γ D as described in Materials and Methods, with visualization of the extracellular (E) secreted material found in the media, the intracellular (I) whole-cell lysates that includes the secreted γ D in the periplasm, and a membrane fraction enriched for ER-derived microsomes (M). The microsome fraction was tested for the presence of N-linked core carbohydrates by processing the sample with endoglycosidase H (EndoH) (+) as described in Materials and Methods. Protein standards are noted to the right in kilodaltons. The HMW ($\blacktriangleright$) and LMW ($\blacktriangleleft$) forms of γ D are indicated.

Degradation of the HMW Aguadilla γ D Is Autophagy-Dependent

We recently demonstrated that the aggregation-prone, misfolded protein A1PiZ^{47,48} forms aggregates within the ER of our yeast model system and that the aggregates are delivered from the ER to the vacuole in an autophagy-dependent manner.¹⁹ Because of their metastable state and the mechanism by which the D domains of fibrinogen γ -chains polymerize in mature fibrinogen,⁷ we suggested that γ D may be aggregation prone and form aggregates within the ER and that clearance of these aggregates may be autophagy-dependent.

To test our hypothesis, the *S. cerevisiae* *atg14Δ* strain was used. Atg14p is a unique protein in a phosphatidylinositol 3-kinase complex that is required for autophagy^{49,50} and is known to be required for the clearance of

Figure 4. Aguardilla γ D degradation is proteasome, vacuole, and autophagy dependent. **A:** Representative colony-blot immunoassay for both the wild-type isogenic parent (WT) and the proteasome mutant (*pre1:2*) strains of *S. cerevisiae* expressing either wild-type γ D (WT γ) or Aguardilla γ D (Ag γ) at low levels off the p426Met25 vector. **B:** Representative immunoblots from a cycloheximide chase analysis of the wild-type isogenic parent (WT), *pep4* Δ vacuolar protease-deficient strain, and *atg14* Δ autophagy-deficient strain of *S. cerevisiae* expressing Aguardilla γ D (Ag γ) at high levels off the p426Gal1 vector. The HMW ($\triangleleft$) and LMW ($\triangleright$) forms of γ D are indicated.

A1PiZ aggregates from the ER.¹⁹ Using cycloheximide chase analyses, we found that HMW Aguardilla γ D was stabilized in the *atg14* Δ strain (Figure 4B). Interestingly, there was less of the LMW Aguardilla γ D at the 0 time point in the *atg14* Δ strain, possibly because there was less Aguardilla γ D that exited the ER via the overflow pathway and transited to the TGN where the pro sequence is cleaved. This may be because the deletion of *ATG14* constitutively induces the unfolded protein response,¹⁹ allowing for more efficient ERAD and removal of the mutant Aguardilla γ D. However, despite the constitutive induction of the unfolded protein response, an accumulation of HMW Aguardilla γ D was seen. Given that defective autophagy leads to the accumulation of A1PiZ aggregates,¹⁹ the stabilization of the HMW Aguardilla γ D in the *atg14* Δ strain suggested autophagy-dependent accumulation of HMW Aguardilla γ D aggregates.

Aggregated HMW Aguardilla γ D Degradation Is Autophagy-Dependent

Two possible intracellular locations for the accumulation of Aguardilla γ D aggregates are suggested from studies of other aggregation-prone ERAD substrates. First, the aggregates may form in the ER and are then delivered to

the vacuole via ER-derived autophagic vesicles, as seen for A1PiZ.¹⁹ Alternatively, Aguardilla γ D aggregates could form in the cytoplasm with subsequent autophagic delivery to the vacuole. For example, cystic fibrosis transmembrane regulator, a known ERAD substrate, is retro-translocated to the cytosol but when inefficiently degraded by the proteasome, forms aggresomes that are cleared by autophagy.^{51–53} Therefore, we asked whether Aguardilla γ D was present in the cytosol or sequestered exclusively within a membrane fraction. High levels of Aguardilla γ D were expressed in the *atg14* Δ strain for 40 hours, after which the cells were collected, and the microsome and cytosol fractions were isolated. Each fraction was examined by immunoblot analysis for the presence of the cytosolic marker protein phosphoglycerate kinase (PGK), an ER luminal marker protein BiP, and fibrinogen. PGK was seen only in the cytosol fraction, as expected. Conversely, HMW Aguardilla γ D along with the ER luminal chaperone BiP resided exclusively within the microsome fraction (Figure 5A). To determine whether the γ D was protected in the lumen of the microsomes or exposed on the exterior of the microsomes, the membrane fractions were treated with trypsin. Trypsin activity was verified by treating microsomes with trypsin in the presence of Triton X-100 to allow trypsin access to both interior and exterior

Figure 5. Autophagy plays a role in clearing aggregated γ D from the ER. **A:** Cytosol (C) and microsome (M) fractions were prepared from the *atg14* Δ strain expressing high levels of Aguardilla γ D as described in Materials and Methods. The microsome fractions were untreated (–) or treated with trypsin (+) or with trypsin and Triton X-100 (++). Fractions were examined by immunoblot analysis using antisera to the cytosolic marker protein, PGK, fibrinogen (Fib), or the ER luminal chaperone, BiP. **B:** Representative immunoblots of fractions from a 5% (top, fraction 1) to 60% (bottom, fraction 20) sucrose density gradient separation of microsome lysates prepared from wild-type (WT) and *atg14* Δ strains expressing high levels of Aguardilla γ D. A short exposure (se) of lane 20 is shown to the right of the blots, and densitometry indicates that there is approximately five times more γ D aggregate in the *atg14* Δ strain compared with that found in the wild-type strain.

Figure 6. Proposed model for Aguadilla γ D quality control. Aguadilla γ D is targeted to ERAD and thus exits the ER by retrotranslocation with subsequent degradation by the proteasome (P). When overexpressed, excess soluble Aguadilla γ D (γ) exits the ER by vesicle transport, transits the Golgi (G), and is sorted to the vacuole (V) for degradation; excess Aguadilla γ D that aggregates ($\gamma\gamma\gamma\gamma$) within the ER is sent to the vacuole via autophagy.

pools of γ D (Figure 5A). These data demonstrate that HMW Aguadilla γ D had not retro-translocated from the ER but rather accumulated within it.

Finally, to address whether the HMW Aguadilla γ D forms aggregates within the ER, lysates of microsomes isolated from both wild-type and *atg14Δ* cells that had been induced to express high levels of Aguadilla γ D were separated by sucrose gradient (see Materials and Methods). Analysis of the wild-type microsomes revealed that some Aguadilla γ D was present in fractions 5 and 6, a density expected for soluble γ D, whereas the majority of the polypeptide was found at the bottom of the gradient in fraction 20, a density expected for aggregated γ D (Figure 5B, WT). The microsomes from the autophagy-defective strain *atg14Δ* contained no detectable soluble Aguadilla γ D and a fivefold greater amount of aggregated γ D in fraction 20 (Figure 5B, *atg14Δ*). Together, these findings demonstrate that Aguadilla γ D aggregates within the ER and that removal of these aggregates is autophagy-dependent.

Discussion

Understanding the molecular mechanisms of cellular protein quality control is important in addressing ER storage diseases, such as the liver disease associated with anti-trypsin deficiency or hypofibrinogenemia. The striking similarities in pathologies of individuals carrying either A1PiZ or the fibrinogen Aguadilla mutation led us to hypothesize that the molecular mechanisms behind the clearance of these aberrant polypeptides would be comparable. To investigate this question, we used our yeast model system and studied the fate of the mutant Aguadilla γ D. A1PiZ had been previously shown to be an ERAD substrate with proteasome-dependent degradation,¹⁸ and here we demonstrate that Aguadilla γ D is also a proteasome-dependent ERAD substrate (Figure 4A). However, when either of these ERAD substrates is ex-

pressed at high levels, such that the ERAD pathway is challenged, overflow pathways act to clear the ER of the excess aberrant polypeptides. One such pathway is used by excess amounts of soluble Aguadilla γ D and soluble A1PiZ, because both transit the secretory pathway and are selectively trafficked from the TGN to the vacuole for degradation (Figure 4B; A1PiZ¹⁹). Furthermore, we have shown that autophagy is required as an additional overflow pathway when excess aberrant polypeptides form aggregates within the ER (Aguadilla γ D in Figures 4B and 5B; A1PiZ¹⁹). It is likely that the decreased amount of LMW Aguadilla γ D seen at the 0 time point in the *atg14Δ* strain is due to the constitutive activity of the unfolded protein response generating increased levels of ER chaperones and elevated ERAD activity.¹⁹ Another possible explanation is that the accumulated Aguadilla γ D aggregates in the ER of the *atg14Δ* strain offer a point for nucleated growth of aggregates, recruiting the majority of the soluble Aguadilla γ D present, thus reducing the pool of HMW Aguadilla γ D reaching the TGN for processing into LMW γ D. Either way, ER quality control must recognize and dispose of two pools of the aberrant Aguadilla γ D in the ER: a soluble pool and an aggregated pool.

These studies provide evidence for the model presented in Figure 6, in which ERAD is the initial step in ER quality control, removing the aberrant Aguadilla γ D for proteasome-dependent degradation. When ERAD is overwhelmed, overflow pathways are used, with the soluble Aguadilla γ D transiting the secretory path and on arrival in the TGN selectively trafficking to the vacuole. In addition, and of importance to the study of ER storage diseases, Aguadilla γ D that aggregates within the ER is cleared from the ER in an autophagy-dependent manner with subsequent vacuolar degradation.

This model, supported by this study and our previous studies with A1PiZ, is directly applicable to the elucidation of the molecular mechanisms of the gain-of-function liver disease associated with hypofibrinogenemia, anti-trypsin deficiency, and perhaps other ER storage diseases. For example, within a population of individuals either homozygous for A1PiZ or heterozygous for fibrinogen Aguadilla, only a subpopulation develops liver disease due to the accumulation of aggregates of aberrant protein in the ER of their hepatocytes.^{10,15,54} Based on our model, it is tempting to speculate that the subsets of individuals who develop liver disease are predisposed to less efficient ER quality control mechanisms, perhaps through a second mutation in a gene important for the prevention or clearance of protein aggregates within the ER. Although we cannot exclude the possibility that other quality control pathways exist, our data suggest that accumulation or autophagy-dependent removal of the aggregates from the ER may be the limiting step that leads to liver disease.

Our model is supported further by the findings from studies of the cellular mechanisms at play in other ER storage diseases. For example, in work to understand the pathology of chronic degeneration and inclusion bodies seen in motoneurons of individuals with amyotrophic lateral sclerosis, Tarabal et al⁵⁵ note a parallel between

α -1-antitrypsin deficiency and the results of their studies. These researchers demonstrated that motoneurons under chronic excitotoxicity display protein aggregation in the ER, autophagic response, and degenerative changes in the motoneurons.⁵⁵ Furthermore, mutant myocilins, associated with autosomal dominant juvenile- and adult-onset primary open angle glaucoma, were shown by Liu and Vollrath⁵⁶ to be highly aggregation prone and to form aggregates in the ER of human trabecular meshwork cells leading to eventual cell death. Finally, autophagy has been shown to play a critical role in the clearance of aggregated mutant vasopressin from the ER, the presence of which leads to the stress-induced neuronal death seen in conjunction with the loss-of-function autosomal dominant familial neurohypophyseal diabetes insipidus.⁵⁷

The number and severity of ER storage diseases points to the importance of understanding the molecular mechanisms underlying the formation and clearance of aggregated aberrant polypeptides within the ER, and yeast model systems provide a means to address these questions and test possible therapeutic agents. Yeast are being used to examine the molecular mechanisms driving the pathology of several other conformational diseases, including cystic fibrosis, prion-related disease, Huntington's disease, and Alzheimer's disease.⁵⁸ Finally, the results presented here and in previous studies with aggregated A1PiZ demonstrate a role for autophagy in clearing aggregated aberrant polypeptides from the ER, findings with important medical applications.

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