Mechanisms Underlying the Cellular Clearance of Antitrypsin Z

Lessons from Yeast Expression Systems

Cristy L. Gelling¹ and Jeffrey L. Brodsky¹

¹Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania

The most frequent cause of α_1 -antitrypsin (here referred to as AT) deficiency is homozygosity for the AT-Z allele, which encodes AT-Z. Such individuals are at increased risk for liver disease due to the accumulation of aggregation-prone AT-Z in the endoplasmic reticulum of hepatocytes. However, the penetrance and severity of liver dysfunction in AT deficiency is variable, indicating that unknown genetic and environmental factors contribute to its occurrence. There is evidence that the rate of AT-Z degradation may be one such contributing factor. Through the use of several AT-Z model systems, it is now becoming appreciated that AT-Z can be degraded through at least two independent pathways. One model system that has contributed significantly to our understanding of the AT-Z disposal pathway is the yeast, *Saccharomyces cerevisiae*.

Keywords: antitrypsin; endoplasmic reticulum-associated degradation; autophagy; yeast

α_1 -Antitrypsin (AT) deficiency (ATD) most commonly arises in individuals homozygous for the Z variant of AT (AT-Z). AT is a member of the serpin family of proteins, many of which are serine protease inhibitors (hence the name), and are aggregation prone. In addition to suffering from chronic lung damage caused by the uncontrolled activity of neutrophil-derived proteases, AT-Z homozygotes are also at increased risk for liver disease (reviewed in References 1 and 2). ATD-associated liver disease results from the retention of the aggregation-prone AT-Z variant at its primary site of production, the endoplasmic reticulum (ER) in hepatocytes.

Although ATD is the most common inherited cause of childhood liver disease, and one of the most common indications for childhood liver transplantation, 8–10% of children who only express the AT-Z protein develop clinically significant liver disease (3). Thus, in most cases, intrinsic cellular defensive mechanisms that act against hepatic accumulation of AT-Z are sufficient to prevent liver disease. However, the large number of cases in which these defenses fail indicates that genetic and environmental factors modify this risk. To date, these modifiers are unknown.

Variations in the efficiency of AT-Z protein degradation may play a key role in determining levels of AT-Z, and potentially disease severity. For example, AT-Z degradation was found to be slower in cells from patients with liver disease compared with those without (4). This lag was specific for the aggregation-prone AT-Z variant, because degradation of a

model misfolded membrane protein was unchanged between these cell lines (5).

PROTEIN QUALITY CONTROL IN THE ER

Given the large size and chemical complexity of proteins, protein folding is inherently error prone. In the event that proteins fail to attain their proper three-dimensional conformations, they may self-associate, forming potentially cytotoxic, insoluble inclusion bodies. To counteract the accumulation of terminally misfolded proteins, protein quality control mechanisms evolved that serve either to help the nonnative species fold or to target the unfolded polypeptide for degradation. The two major mediators of protein degradation in the cell are the proteasome, a multicatalytic protein complex located in the cytosol, and the lysosome, an acidic organelle that harbors a number of hydrolytic enzymes.

Approximately one-third of all proteins, including AT, are secreted or membrane proteins, and thus associate with the ER at some point in their lifetime (6, 7). Early studies demonstrated that misfolded ER proteins were selectively degraded, and it was suggested that an ER luminal protease was responsible for this critical event in ER quality control (8). Nevertheless, for many years, the factors required for ER protein degradation remained uncharacterized.

To better define how aberrant ER proteins are selected and degraded, we developed an *in vitro* system in which the biogenesis of a model mutated, secreted protein could be followed in ER-derived vesicles prepared from yeast. We discovered that the mutated protein was exported from the ER vesicles into the cytosolic fraction, an event that required ER luminal chaperones (9). We and others subsequently found that the degradation of these “retro-translocated” ER proteins was mediated by the cytosolic proteasome, a 26S multicatalytic protease that had been previously found to trigger the destruction of misfolded cytoplasmic proteins (10, 11). We termed this process ER-associated degradation (ERAD). Next, to generalize these findings, we employed a yeast expression system that McCracken and Kruse (12) had developed to monitor the fates of AT-Z and the wild type AT-M species. As anticipated, the degradation of AT-Z was significantly delayed in yeast strains that were defective for proteasome function. In parallel to our efforts, the Perlmutter laboratory (13) discovered that the degradation of AT-Z in the mammalian ER was also proteasome-mediated. Together, these studies implicated the ERAD pathway as a major contributor to AT-Z quality control.

Further studies in the yeast system led to additional insights into the AT-Z degradation pathway. For example, we found that mutations in the ER luminal chaperone, immunoglobulin heavy chain binding protein (BiP), slowed the ERAD of AT-Z (14) (Figure 1). A role for BiP in AT-Z degradation was later corroborated in mammalian cells (15, 16). We also reported that yeast strains lacking a conserved proteasome assembly chaperone, Add66 (alpha-1 proteinase inhibitor degradation deficient-66), exhibit reduced proteasome activity, and thus accumulated

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Correspondence and requests for reprints should be addressed to Jeffrey L. Brodsky, Ph.D., A320 Langley Hall, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260. E-mail: jbrodsky@pitt.edu

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Figure 1. Endoplasmic reticulum (ER)-associated degradation of α_1 -antitrypsin (AT). The ER luminal chaperone, immunoglobulin heavy chain binding protein (BiP), also known as Kar2 (Karyogamy-2) in yeast, (in pink) is involved in the recognition and targeting of misfolded luminal proteins for degradation. AT (in green, with red N-glycan modifications) physically interacts with BiP, and BiP is required for the efficient ER-associated degradation (ERAD) of AT. Although not shown in this figure, some luminal components that constitute the glycan quality control machinery (e.g., calnexin, mannosidase, and ER degradation-enhancing α -mannosidase-like protein, or EDEM) have also been shown to facilitate AT turnover in mammalian cells (20). AT that is to be degraded is retrotranslocated across the ER membrane to the cytosol. Ubiquitination of ERAD substrates typically occurs at the ER membrane, but, in the case of AT, this has not yet been reported. Proteasome activity is also required for the ERAD of AT. In the absence of the yeast proteasome-assembly chaperone, Add66 (human proteasome assembly chaperone-2, or PAC2), 15S “half-particle” proteasomes accumulate, and the efficiency of AT degradation is decreased (17).

AT-Z (17). Additional work on AT-Z and other AT variants, most notably null Hong Kong, has similarly led to new insights into the factors that select misfolded forms of AT for the ERAD pathway in mammalian cells (18). Recently, a single nucleotide polymorphism affecting expression of ER mannosidase I—a key mediator of ERAD—has been proposed to represent a predictor for the age of onset of end-stage liver disease in ATD (19). The mannosidase acts as a “timer” for the degradation of ER-retained, glycosylated ERAD substrates, such as AT. However, because AT-Z degradation efficiency correlates with disease severity (4), and based on the existence of many other factors that modulate ERAD efficiency (20), we suggest that additional genetic modifiers associated with ATD-associated liver disease will be linked to the ERAD pathway.

TABLE 1. SELECT PROTEINS OF THE CORE AUTOPHAGY MACHINERY IN YEAST AND THEIR HOMOLOGS IN MAMMALS

Functional Class	Yeast Protein	Mammalian Protein*
Atg1-kinase complex	Atg1	ULK1/2
	Atg13	mAtg13
	Atg17	FIP200
	Atg29	—
	Cis1	—
Class III phosphatidylinositol 3-kinase complex I	Vps34	hVps34
	Vps15	p150
Atg9 complex and related	Vps30/Atg6	Beclin1
	Atg14	Atg14L
	Atg9	mAtg9
	Atg2	Atg2A/B
Atg12 conjugation system and Atg16 complex	Atg18	WIPI-1
	Atg12	Atg12
	Atg5	Atg5
Atg8 activation and conjugation system	Atg7	Atg7
	Atg10	Atg10
	Atg16	Atg16L
	Atg8	LC3
	Atg3	Atg3
	Atg4	Atg4B
	Atg7	Atg7

Definition of abbreviations: Atg = autophagy related; Cis = Clk1 suppressing; FIP = focal adhesion kinase family interacting protein of 200 kD; hVps = human Vps; LC = microtubule associated protein 1 light chain 3; mAtg = mammalian Atg; Ulk = unc-51-like kinase 1; Vps = vacuolar protein sorting; WIPI = WD-repeat protein interacting with phosphoinositides.
* Dashes indicate that a homolog has not been identified.

AUTOPHAGY: A COMPLEMENTARY PATHWAY FOR PROTEIN DEGRADATION

A second route by which ER-localized proteins can be degraded is macroautophagy, which is here referred to as autophagy. Most of the molecular underpinnings of this pathway were first established in yeast, and nearly all of the components required for autophagy are conserved between yeast and man (21) (also see Table 1). In short, autophagy results in the trafficking of organellar and cytosolic cargo to the lysosome (or, in yeast, the vacuole). In this compartment, the contents of autophagic vesicles are released and destroyed by resident proteases (Figure 2A). Autophagic cargo can be selectively or nonselectively encapsulated by cup-shaped vesicles, known as isolation membranes or phagophores, which close to form double-membrane autophagosomes. The source of the membrane for autophagosomes has been a topic of investigation since the first morphological descriptions of autophagy, yet remains contentious.

The core machinery required for the formation of autophagosomes in yeast has been classified into five classes or complexes: the Atg1 (autophagy related-1) kinase and its regulators; an autophagy-specific class III phosphatidylinositol 3 (PI3)-kinase (PI3K) complex; the Atg9 complex; and the Atg12, and the Atg8 ubiquitin-like conjugation systems (21) (Table 1).

In yeast, the earliest known step in autophagosome formation is the assembly of a single proteinaceous preautophagosomal structure (PAS) adjacent to the vacuole. The PAS is thought either to mature into an isolation membrane or to be the site from which isolation membranes are produced. It is unknown whether mammalian cells have a similar structure, but isolation membranes are apparently formed at multiple sites. However, in both yeast and mammals, PI3-phosphate (PI3P) is required for autophagy. Recent studies in mammalian cell

Figure 2. Autophagy and the class III phosphatidylinositol 3-kinase (PI3K) complexes. (A) The formation of cup-shaped isolation membranes (IM) is preceded by formation of the proteinaceous preautophagosomal complex (PAS). Closure of the IM results in a double-membrane vesicle called the autophagosome. Fusion of the outer membrane of the autophagosome with the vacuole/lysosome releases a single membrane-bound autophagic body (AB) that is degraded by vacuolar hydrolases. (B) Class III PI3K complexes convert phosphatidylinositol to PI3-phosphate. Complex I is required for autophagy, most likely for recruitment of autophagy proteins to the site of autophagosome formation. In yeast, complex II is required for the Golgi CPY-to-vacuole pathway. Complexes I and II are distinguished by the presence of the Atg14 (autophagy related-14) and Vps38 (vacuolar protein sorting-38) proteins (in blue and green, respectively), which may mediate the different locations of the two complexes. Vps34 is the catalytic subunit and Vps15 tethers the complex to membranes, but the molecular function of Vps30/Atg6 is unknown.

culture showed that induction of autophagy stimulated the formation of PI3P-rich compartments at specific sites within the ER membrane (22). These compartments formed dynamic ER-connected structures, termed “omegasomes,” inside which Atg5 and microtubule associated protein 1 light chain 3 (LC3, the mammalian homolog of Atg8) accumulated before being released. The authors proposed that the omegasome is an ER membrane-contiguous platform for the assembly of autophagosomes.

In yeast, PI3P is generated by Vps34 (vacuolar protein sorting-34), the catalytic subunit of an autophagy-specific PI3K complex (complex I) and a vacuolar sorting PI3K complex (complex II) (23). Both complexes also contain Vps30/Atg6 and the Ser/Thr kinase Vps15, and hence are distinguished by a single unique subunit: Atg14 in the case of complex I (autophagy) and Vps38 in the case of complex II (vacuolar sorting) (Figure 2B). The mammalian counterpart of complex I is required for autophagy, and the counterpart of complex II is localized to early endosomes (24). The function of complex I is therefore conserved from yeast to mammals, although the functional conservation of complex II is still unclear.

Atg8/LC3 is a ubiquitin-like protein that becomes conjugated onto phosphatidylethanolamine (PE) (25). Atg8 is first proteolytically activated by Atg4, and subsequently conjugated to PE by the combined activities of the Atg7 and Atg3 enzymes. The Atg5/Atg12/Atg16 complex may further catalyze this modification. Atg8-PE plays a vital role in PAS/isolation membrane expansion. Its concentration is limiting for autophagosome size (26), and Atg8-PE stimulates liposome hemifusion *in vitro* (27). This suggests that Atg8-PE could facilitate lipid incorporation into the expanding membrane. However, evidence has recently emerged for an alternative form of autophagosome maturation that is independent of the Atg5/Atg7/Atg8 system, and appears to involve fusion with trans-Golgi/late-endosome-derived vesicles (28). Further “twists” on the autophagy paradigm are sure to emerge, as new targets of this pathway are continuously being discovered (*see* subsequent discussion here).

SELECTIVE AUTOPHAGY

The core machinery for nonselective autophagy can also be utilized for selective autophagy, during which specific proteinaceous and organellar cargoes are captured and destroyed. The yeast cytoplasm-to-vacuole pathway represents the canonical example of selective autophagy. Specific oligomerized cargo proteins in the cytosol bind the Atg19 receptor, which, in turn, binds the Atg11 adaptor (29, 30). Once transported to the PAS, this cytoplasm-to-vacuole complex binds Atg8, resulting in encapsulation within the forming autophagosome.

Several examples of selective autophagy of organelles are also known. Selective autophagy of mitochondria (“mitophagy”) has been shown to target damaged mitochondria (31). The autophagosomal engulfment of portions of the ER membrane (“ERphagy”) has been demonstrated in yeast cells undergoing homeostatic remodeling of ER volume (32, 33).

Another form of selective autophagy is the destruction of proteinaceous aggregates in the cytosol (“aggrephagy”). During aggrephagy, misfolded or damaged ubiquitin-marked proteins form cytosolic inclusions that, in mammals, are recognized by p62, a protein that self-associates to promote inclusion formation (34). p62 specifically binds LC3 to recruit the isolation membrane (35, 36).

AUTOPHAGY AND THE DESTRUCTION OF THE Z VARIANT OF ANTITRYPSIN

The first hint that ER-retained forms of AT-Z might be degraded by autophagy was provided by an observed increase in autophagosome formation in AT-Z-expressing cell culture and mouse models, as well as in the livers of patients with ATD (37, 38). Subsequently, AT-Z degradation was found to be delayed in autophagy-deficient mouse embryonic fibroblasts. Consequently, AT-Z accumulated in large, insoluble inclusions. AT-Z in wild-type mouse embryonic fibroblasts was also observed to partially colocalize with autophagosomes when lysosome fusion was impaired (39). These data indicate that autophagy represents a second route by which AT-Z can be degraded. Recent studies examining the fate of another secreted serpin implicate both autophagy and ERAD as contributing to its degradation (40).

Using the yeast AT-Z expression system, we tested the hypothesis that the “decision” between the ERAD versus autophagic route for AT-Z degradation reflected the amount, and therefore aggregation propensity, of AT-Z. Indeed, we discovered that, at low levels of expression, AT-Z remained largely soluble, and could be handled by the ERAD pathway. In contrast, higher levels of AT-Z resulted in the formation of polymers, and a functional autophagic pathway was required for degradation and cell viability (41). As a result, the trigger for induction of autophagy by AT-Z might be the formation of AT-Z polymers. In mammalian cells, the AT-Z-induced response to aggregates may be mediated in part by the regulator of G signaling 16 protein. Regulator of G signaling 16 protein is an inhibitor of an autophagy-repressing G protein, Gαi3, and is specifically induced by AT-Z in cell and mouse models, and in the livers of patients with ATD (42).

An important therapeutic question arising from these data is whether small-molecule modulators that dissolve AT-Z polymers and/or inducers of AT-Z autophagy might offset the

catastrophic consequences of AT-Z-induced liver damage. Indeed, a compound that decreased AT-Z polymerization *in vitro* also increased the rate of AT-Z degradation in cell lines (43), and an inducer of autophagy was recently shown to decrease liver damage in an AT-Z mouse model (44).

MITOPHAGY AND ANTITRYPSIN DEFICIENCY

AT-Z expression results in mitochondrial damage and extensive mitochondrial autophagy (45). This may be caused by a hyperactive autophagic response to AT-Z. However, it is unknown whether induction of autophagy can lead to “off-target” damage to otherwise functional mitochondria. If the cell is capable of responding to AT-Z by selective ERphagy, the extensive mitophagy observed may be a consequence of mitochondrial damage, rather than a cause. Interestingly, mitochondria were recently shown to contribute membrane to starvation-induced autophagosomes (46).

THE HUNT FOR GENETIC MODIFIERS OF ANTITRYPSIN DEFICIENCY

The discovery of genetic modifiers of human disease has primarily been accomplished through laborious and costly correlation studies. To surmount these barriers, we instead proposed that modifiers might be identified through the development of yeast expression systems for proteins linked to specific diseases. Yeast genetics and genomic methods could then be used to identify conserved factors that impact the protein's properties associated with disease presentation. To identify genetic modifiers of ATD, we sought to identify and characterize conserved yeast genes that alter AT-Z levels, and, in turn, its polymerization. Two approaches have been taken toward this goal.

The first approach was a classic genetic screen, in which mutagenized yeast colonies were surveyed for those that accumulated higher-than-background levels of AT-Z (47). One of the mutants identified was shown to have a defect in the gene encoding the PI3K complex I and II component, *Vps30/Atg6* (41). The increased AT-Z levels observed in the *vps30* mutant was due to a degradation defect, and a similar phenomenon was observed in both *atg14* and *vps38* mutants, which have defects in the autophagy-specific complex I and carboxypeptidase Y-to-vacuole (CPY-to-vacuole) sorting complex II, respectively. This indicates that AT-Z can be degraded in the vacuole after delivery by at least two routes: autophagy and vesicular transport via the CPY-to-vacuole pathway. Interestingly, an *atg13* mutant degraded AT-Z with wild-type kinetics; therefore, the regulatory *Atg1/Atg13/Atg17* complex is not required for this process. This result indicates that AT-Z degradation via the autophagic pathway is independent of *Atg13* phosphorylation by the Tor kinase, as occurs during starvation. Consequently, the degradation of AT-Z is unlikely to result from a generalized stress response.

In the second approach to finding novel modifiers of AT-Z biogenesis, a targeted screen was performed on strains lacking uncharacterized genes that are otherwise induced by the unfolded protein response (48). The rationale behind this attack was that many unfolded protein response-targeted genes are required for ERAD. Among the examined genes that, when mutated, led to increased levels of AT-Z was a previously uncharacterized, but conserved, proteasome assembly chaperone, which was discussed previously here (17). The contributions of other “hits” from this screen on AT-Z expression and degradation are currently being examined.

Together, these data demonstrate that the autophagic pathway can serve as a backup system for ERAD to help prevent

the accumulation of AT-Z polymers. The need for this overflow pathway for AT-Z may be vital, because substrate retrotranslocation to the cytosol for ERAD would likely be less efficient for AT-Z polymers than for soluble AT-Z. Two recent publications support this hypothesis. First, a panel of misfolded cytosolic proteins expressed in yeast partitioned between two different compartments, depending upon their solubility and ubiquitination state (49). Soluble, polyubiquitinated proteins accumulated in a juxtanuclear ER membrane focus that colocalized with proteasomes. Insoluble, nonubiquitinated proteins accumulated, instead, in a perivacuolar compartment that colocalized with *Atg8*. Second, the degradation pathway for the cystic fibrosis transmembrane conductance regulator (CFTR) was shown to correlate with its aggregation state in yeast (50): Whereas one population of an EGFP-CFTR fusion protein exhibited diffuse staining in the ER and was degraded by the proteasome, a distinct protein pool accumulated in ER-resident foci. These foci were cleared by autophagy, and contained immobile CFTR, as determined by fluorescence recovery after photobleaching analysis.

CONCLUSIONS

As should be evident from this article, data from the yeast AT-Z expression system have helped complement studies in higher cell types and, in some cases, have even raised novel insights into the mechanisms by which AT-Z is targeted for degradation. Moreover, other proteins linked to human diseases might be subject to both ERAD and autophagy during cellular quality control “decisions” (50). Our hope is to continue to co-opt the yeast system to define how previously ill-characterized or novel factors impact AT-Z biogenesis. Results from these efforts can then be confirmed in mammalian cell systems. Ultimately, targets from this analysis may serve as new avenues for therapeutic intervention in ATD.

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